Notch and CD4+ T cell function

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Abstract

$CD4^+$ T cells require heterogeneity in effector responses to respond effectively to the diversity of pathogenic challenge. The fate decisions made by naïve $CD4^+$ T cells occur on first encounter with antigen. Activation via the T cell receptor (TCR) selects for antigen specificity while the influence of TCR/MHC/peptide avidity and co-stimulatory signals via CD28, CD40L, OX40, ICOS, CTLA-4, and cytokines determine differentiation into Th1/Th2, memory or regulatory/anergic cells. The identification of components of the Notch signalling pathway, central to cell fate determination in embryogenesis, in the mammalian immune system has expanded current thinking on the specification of effector cell phenotype. This thesis was thus designed to test the hypothesis that Notch signalling is able to influence $CD4^+$ T cell effector function.

Activation of purified murine $CD4^+$ T cells enhanced expression of the Notch target gene *hes1* and induced differential expression of Notch receptors and ligands compared to unstimulated cells. Surface staining for Notch1 revealed that unstimulated cells expressed this receptor at the membrane. Activation of T cells induced capping of Notch1, which was found to co-localise with CD4.

Capping of surface Notch and co-localisation with CD4 upon T cell activation was reminiscent of immunological synapse formation, suggesting that Notch may interact directly with T cell receptor signaling. Notch signalling was attenuated in $CD4^+$ T

cells using a γ-secretase inhibitor, allowing assessment of Notch function in determining effector function. Notch signal inhibition in the context of anti-CD3-Ab stimulation, resulted in inhibition of TNFα, IFNγ, IL-4 and IL-10 secretion. Blockade of Notch signalling where cells were stimulated with anti-CD3/28-Ab did not down-regulate TNFα, IFNγ or IL-4 secretion, but IL-10 was inhibited. Notch signalling may thus be important as a co-stimulator when CD28 signalling is limiting. Of note is the requirement of Notch signalling in the induction of the immunoregulatory cytokine IL-10, known to be related to T reg cell activity.

Naturally occurring $CD25^{+}CD4^{+}$ T regulatory cells and *in vitro* generated Tr1 cells were examined for expression of Notch pathway components to ascertain if Notch may be involved in mediating regulatory function. Both populations were found to express higher levels of the Notch ligand Delta1 than non-regulatory $CD4^{+}$ T cells. Inhibition of Notch signalling did not affect the ability of $CD25^{+}CD4^{+}$ T cells to regulate proliferation of $CD4^{+}CD25^{-}$ T cells, but did reduce secretion of IL-10.

These findings indicate that Notch receptors and ligands are expressed by $CD4^{+}$ T cells. Notch may also be a component of the immune synapse and potentially modulates $CD4^{+}$ T cell effector function through regulation of cytokine secretion, particularly IL-10.

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Robert Alexander Benson

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Dedication

To my family

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Abbreviations

7AAD	7-aminoactinomycin
Ac/Sc	Achaete/Scute complex
Ab	antibody
Ad	adenovirus
APC	antigen presenting cell
APP	amyloid precursor protein
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
BM	bone marrow
C. elegans	*Caenorhabditis elegans*
Ca^{2+}	calcium ion
CBF-1	C promoter binding factor-1
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary DNA
CLP	common lymphoid progenitor
CMV	cytomegalovirus
CSL	CBF-1, Su(H), LAG-1
CTL	cytotoxic T cell
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
DAG	diacylglycerol
DC	dendritic cell
Der p	Dermatophagoides pteronyssinus
Der p1	Der p type 1 allergen
DN	CD4 CD8 double negative
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dioxynucleotide triphosphate
DP	CD4 CD8 double positive

EAE	experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
EBNA2	EBV nuclear antigen 2
EDTA	Ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbant assay
Erk	extracellular signal receptor regulated kinase
E(spl)	enhancer of split
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gravitational force
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony stimulating factor
GSK	glycogen synthase kinase
hes	hairy/enhancer of split
ICAM	intercellular adhesion molecule
ICD	intracellular domain (of Notch)
ICOS	inducible costimulator
IFNγ	interferon-gamma
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
Jnk	Jun N terminal kinase
kb	kilobases
LFA	lymphocyte functional antigen
LPS	lipopolysaccharide
MACS	magnetic cell sorting
MCMV	murine cytomegalovirus
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
MOI	multiplicity of infection

mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappaB
NICD	notch intracellular domain
NICD1	notch1 intracellular domain
NICD3	notch3 intracellular domain
NK	natural killer cell
°C	degrees centigrade
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	phosphoinositide
PKC	protein kinase C
PLCγ	phospholipase C gamma
RAM	RBP-J association molecule
RANK	receptor activator of NFκB
RBC	red blood cell
RBP-Jκ	recombination-signal binding protein Jκ
RIP	regulated intramembrane proteolysis
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SLP-76	src-homology2-domain containing leukocyte phosphoprotein of 76kD
SMAC	supra-molecular cluster (c or pSMAC: central or peripheral SMAC)
SP	single positive
TAMRA	6-carboxytetramethylethylenediamine
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper
Ta	annealing temperature
TAE	Tris acetate and 10mM EDTA

T-ALL	T acute lymphoblastic leukaemia
TNF	tumour necrosis factor
Tr1	Type 1 regulatory T cell
TRANCE	TNF-related activation induced cytokine
T reg	regulatory T cell
μg	microgram
μm	micro metres
wt	wild type
ZAP-70	zeta associated protein of 70kD

1 Introduction

The mammalian immune system consists of an array of cells evolved to provide protection against pathogens. Flexibility within the immune system allows differentiation between harmful and innocuous antigens, and cell fate decisions have to be made to produce the appropriate response. Although the innate immune response is crucial in preventing infection, the adaptive response provides specificity and memory, enhancing the overall efficiency of the host defence mechanisms.

Dendritic cells (DCs) act to bridge innate and adaptive immunity, transmitting signals received directly from pathogens and inflamed tissues to $CD4^+$ T cells that translate this information into a response specific for the pathogen. Once the decision to respond to specific antigen is made, $CD4^+$ T cells proliferate and differentiate into effector cells. Development of an appropriate $CD4^+$ T effector phenotype is crucial in determining the outcome of a response directing adaptive immunity and augmenting innate responses. Circumstantial evidence suggests that Notch signalling may influence the role $CD4^+$ T cells play in an immune response.

The Notch pathway is classically associated with cell fate choices during development, however, the effects of Notch signalling extend beyond embryonic patterning and into regulation of the mature immune system. Notch signalling regulates differentiation of macrophages, dendritic cells (DCs), and lymphocytes. A large body of evidence details the critical role of Notch in controlling thymocyte

development where it promotes T versus B lymphocyte lineage commitment, determines T cell receptor usage and influences expression of CD4 and CD8.

Given its importance in the development of $CD4^+$ T cells, it was hypothesised that Notch signalling has a role in differentiation of the mature, peripheral $CD4^+$ T cell. By influencing the effector profile/Th phenotype of activated $CD4^+$ T cells, Notch signalling may provide an important link establishing an immune response.

On beginning work for this thesis, the only direct evidence linking Notch signalling and $CD4^+$ T cell function was derived within our own laboratory. Induction of Notch signalling *in vivo* was capable of generating a population of $CD4^+$ T cells capable of regulating immune responses to house dust mite allergens (1). The aim of this thesis was to clarify the role of Notch signalling in relation to $CD4^+$ T cell function. *In vitro* studies were used to establish whether Notch has a role in normal $CD4^+$ T cell responses. By means of an introduction, background information regarding the activation and differentiation of $CD4^+$ T cells is detailed. An overview of the Notch signalling pathway is also provided and is followed by information pertaining to Notch activity in T lineage cells.

1.1 Activation of $CD4^+$ T cells

T cell activation is dependent upon transduction of specific surface receptor interactions into new gene transcription and translation. $CD4^+$ T cell activation follows recognition of specific antigenic peptides presented in the context of MHC class II (signal 1) and additional co-stimulation (signal 2) received from professional antigen presenting cells (APCs). Although both macrophages and B cells are capable of presenting antigen to $CD4^+$ T cells, it is believed that the main APCs responsible for priming naïve T cells are dendritic cells (DCs). Antigen bearing DC migration to lymphoid tissue allows naïve $CD4^+$ T cells to sample peptide/MHC class II complexes by means of a clonally-specific antigen-binding surface receptor, the T cell receptor (TCR). The major source of costimulatory signalling is accepted to be via CD28 ligation by either CD80 or CD86 (B7.1, B7.2). However, a number of other costimulatory molecules are involved in focussing the interaction between T cell and APC and the modulation of subsequent gene expression.

1.1.1 T cell receptor signalling (signal 1)

The TCR complex consists of a multichain complex of six or more distinct polypeptides (Figure 1.1). The clonotypic antigen recognition portion (TCR) of the complex consists of a polymorphic α and β chain heterodimer, lacking signalling capability. Signal transduction is reliant on the invariant CD3 complex. CD3 associates with the TCR$\alpha\beta$ as an ε chain dimerised with either γ or δ. Each chain contains a single ITAM (immunoreceptor tyrosine-based activation motif). An additional six ITAMs are present on the ζ chain homodimer (Figure 1.1).

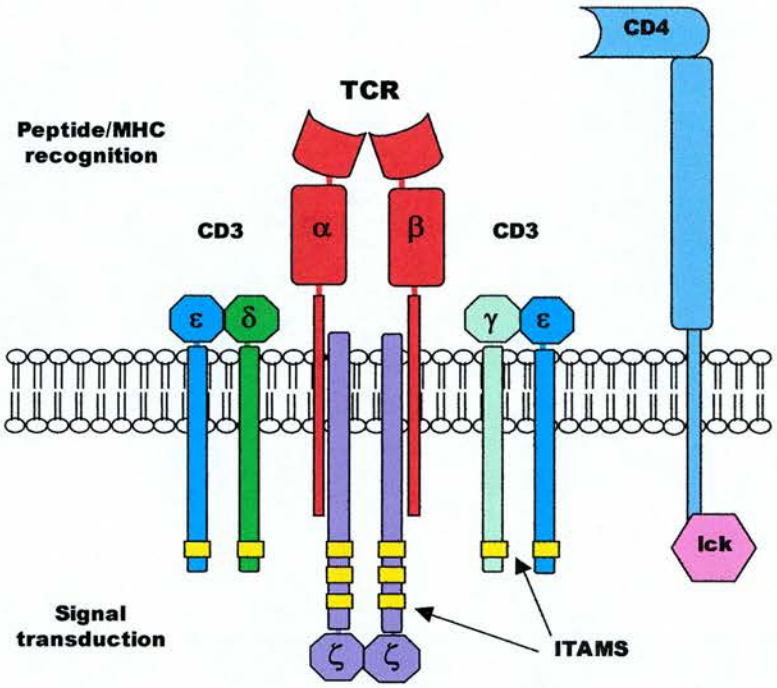


Figure 1.1: The T cell receptor and co-receptor complex. The disulphide linked α and β chains of the TCR (red) are incapable of mediating signal transduction. Association with the CD3 complex (possibly through ionic interactions) links antigen reception to signal transduction. The CD3 complex consists of a εδ dimer, εγ dimer and a mainly cytoplasmic ζζ homodimer (purple). It is currently believed that two TCR αβ dimers associate with a single CD3 complex, balancing ionic charges of the transmembrane regions. Receptor ligation induces phosphorylation of immunoreceptor tyrosine-activation motifs (ITAMs) (yellow) by Src kinases, allowing binding and activation of signalling molecules. The Src kinase fyn can be found associated with the TCR. Another vitally important Src family member, lck, is recruited by the co-receptor CD4 (blue) which binds directly to the MHC class II molecule.

T cell/APC interactions are initially mediated by adhesion molecule interactions, allowing sampling of peptide/MHC class II complexes by the TCR (Figure 1.2A). Recognition of specific ligand induces rapid organisation of surface receptors at the point of contact between T cell and APC to form an immunological synapse (IS). The IS is a three dimensional structure consisting of a "bull's eye" arrangement of supramolecular clusters (SMACs) (Figure 1.2B) (2). Classically, central

supramolecular cluster (cSMAC) and peripheral supramolecular cluster (pSMAC) contain TCR/MHC and adhesion molecules respectively. Such organisation allows concentration of signal transduction molecules associated with lipid rafts, increasing sensitivity to antigen. Blockade of IS formation prevents full T cell activation. Induction of strong TCR signalling is associated with rapid dissolution of the IS and cessation of signal transduction. So the IS may also function to prevent excessive T cell stimulation.

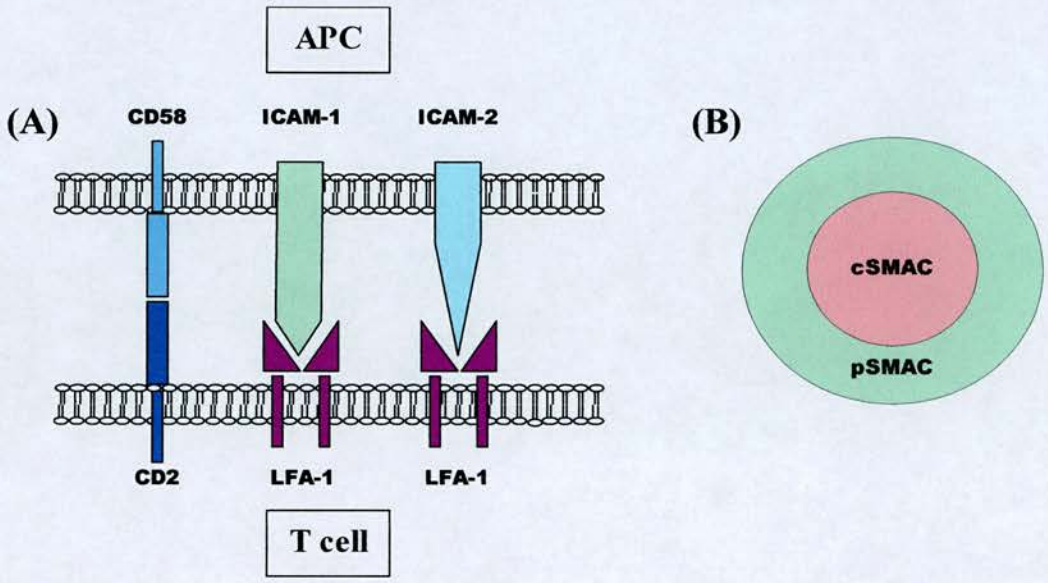


Figure 1.2: Interaction between APC and T cell. Naïve $CD4^+$ T cells follow chemotactic gradients to reach the T cell dependent areas of secondary lymphoid tissues. Chemokine reception induces polarisation of adhesion molecules and TCR complexes to the leading edge of the T cell positioning surface receptors for APC/T cell interaction. Initial foci develop consisting of adhesion molecules such as LFA-1 and CD2 (A), providing time for TCR/MHC interactions to occur. Induction of TCR signalling is followed by a conformational change in LFA-1, enhancing its affinity for ICAMs expressed by the APC. This event is rapidly followed by organisation of surface molecules such as the TCR, CD4, CD28, CD2 and LFA-1 into the immunological synapse (IS). This structure concentrates the signal transduction machinery in the central supramolecular cluster (cSMAC), and adhesion molecules to the peripheral SMAC (pSMAC) (B). IS formation augments T cell activation by focusing the interaction between the APC and T cell. Release of cytokines into the IS may also allow directed polarisation of T cell effector function by APCs.

The pivotal point in T cell activation seems to be the transduction of binding a specific peptide/MHC class II complex into increased phosphorylation of the CD3 ITAMs (immunoreceptor tyrosine-based activation motifs) particularly those of the ζ chain (Figure 1.3). The mechanism leading to this has not yet been established but a combination of recruitment of lck by co-ligation of CD4, IS concentration of src kinases and target molecules, and possible exclusion of negative regulators (such as Csk) may be responsible (1-3). Enhanced ITAM phosphorylation allows recruitment and activation of zeta-associated protein-70 (ZAP-70). ZAP-70 is responsible for the phosphorylation of the palmitoylated adaptor protein LAT (linker of activation in T cells) and of SLP-76 (SH2-domain-containing leukocyte protein of 76kDa) (Figure 1.3)

Phosphorylation of LAT and SLP-76 allows recruitment of Tec kinases and GEFs (guanine-nucleotide exchange factors) leading to the activation of PLCγ (phospholipase C-γ) and the Ras/Raf MAPkinase cascade respectively (Figure 1.3) (4, 5). Activation of these two pathways in concert generates functional NFAT (nuclear factor of activated T cells), NFκB (nuclear factor κB) and, in the context of appropriate co-stimulation, AP-1 (activator protein-1) (2, 5). The result is initiation of new gene transcription, culminating in clonal expansion and development of effector function.

Activated PLCγ converts membrane PIP2 (phospho-inositol-biphosphate) into IP3 (inositol-triphosphate) and DAG (diacyl-glycerol), culminating in NFAT and NFκB translocation to the nucleus (Figure 1.3) (3). IP3 allows release of intracellular

calcium stores, required for calmodulin dependent calcineurin activation. Calcineurin is a serine/threonine phosphatase that dephosphorylates the nuclear localisation sequence of cytoplasmic NFAT allowing its nuclear translocation. DAG is required for the activation of PKC (protein kinase C). This occurs directly as with nPKC (novel PKC) or in conjunction with Ca^{2+} as for cPKC (conventional PKC). PKC activation of IκK (IκB kinase) leads to the removal of IkB from NFκB, allowing nuclear localisation of the active transcription factor.

AP-1 consists of dimers of Jun and Fos. Transcription of Fos is induced by TCR activation of the Ras/Raf MAPkinase pathway (Figure 1.3) (3). Association of the adaptor protein Grb2 with LAT/SLP-76 recruits the GEF Sos which activates the small G-protein Ras. Ras in turn stimulates the MAP kinase kinase kinase Raf which subsequently triggers Mek1 and 2 activation of Erk1 and 2. Erk MAPkinase is responsible for increased transcription of Fos through stimulation of the transcription factor Elk. However, Jun is also required to generate active AP-1.

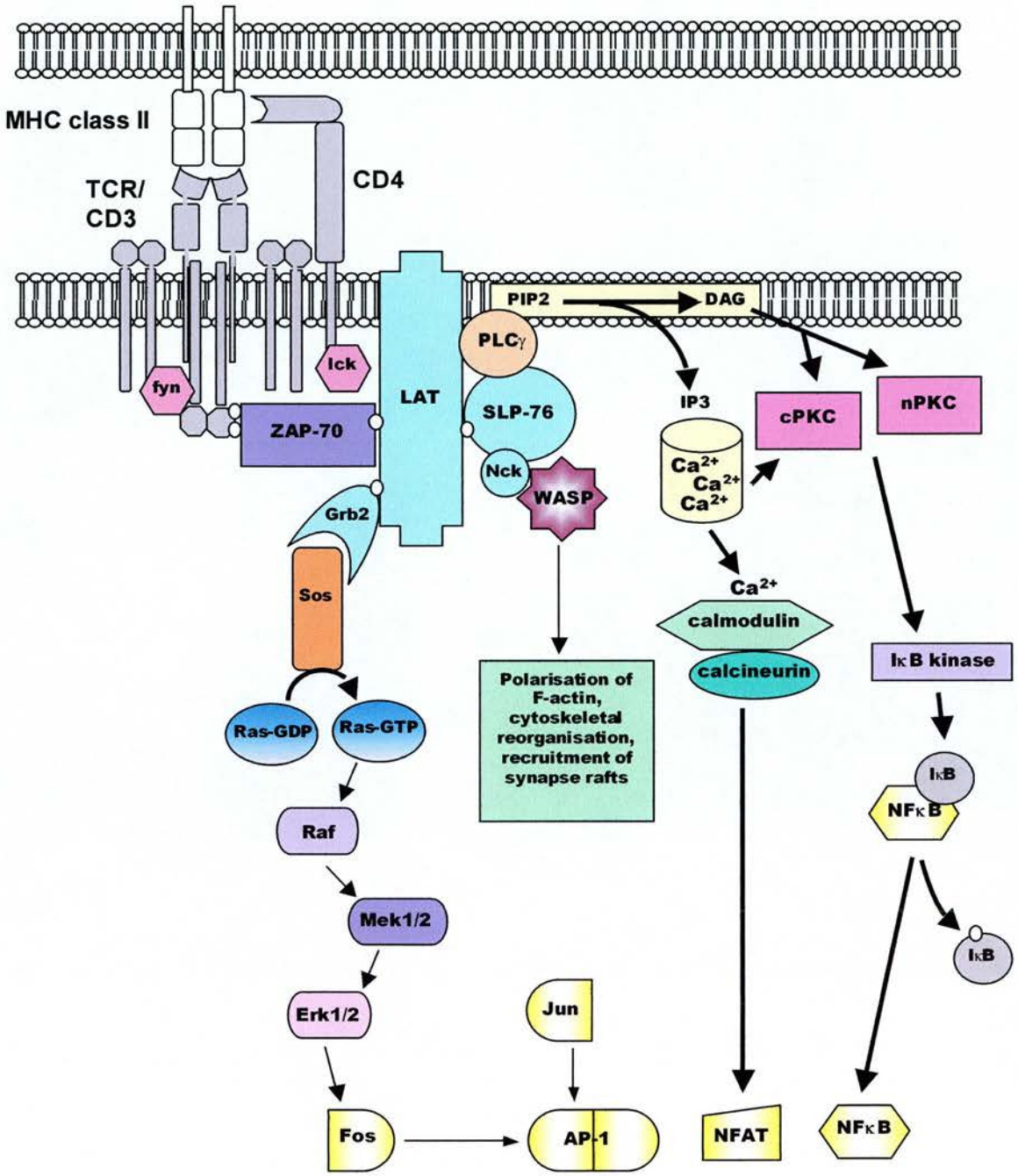


Figure 1.3: TCR signal transduction. Ligation of the TCR and CD4 by specific peptide/MHC class II stimulates Src kinase activity (lck and fyn), leading to ITAM phosphorylation, recruitment and activation of ZAP-70. Subsequent phosphorylation of the adaptor proteins LAT and SLP-76 stimulate MAPkinase cascades (Ras/Raf), cytoskeletal reorganisation, increased calcium concentrations and PKC activity. Stimulation of these pathways leads to induction of gene expression mediated by the transcription factors AP-1, NFAT and NFκB.

1.1.2 Costimulation (signal 2)

TCR stimulation is generally regarded as being insufficient for naïve T cell priming, with antigen presentation in the absence of costimulation resulting in anergy or apoptosis. TCR stimulation in the absence of costimulation results in fyn hyperphosphorylation of Cbl and generation of active Rap1 by the Cbl/CrkL/C3G complex (3). Subsequent sequestration of Raf by Rap1 prevents activation of the Ras/Raf MAPkinase cascade, blocking TCR mediated IL-2 induction. Increased calcium flux promotes high levels of NFAT in the absence of AP-1 activity, again blocking IL-2 gene transcription. It has also been recently suggested that NFAT on its own may induce expression of specific anergy associated genes (6).

Approximately 95% of $CD4^+$ T cells express CD28. TCR and CD28 co-ligation prevents anergy induction by promotion of lck rather than fyn activity. CD28 signalling is also a major source of active Jun. Binding of CD28 to ligand (CD80/86) triggers a second MAPkinase cascade induced by recruitment of the GEF, Vav, to LAT/SLP-76. The generation of Rac-GTP (Rho family member of small G-proteins) is followed by sequential activation of Mekk, Jnkk and then Jnk. Phosphorylation of Jun then allows dimerisation with Fos and the generation of AP-1.

The critical requirement for CD28 signalling has been a subject of much debate. T cells from CD28 deficient mice are still capable of being activated, secreting IL-2 and dividing (7). This may be due to signals received from other costimulatory molecules such as ICOS (8) or as yet unidentified surface receptors. The idea of a strict two-signal model of T cell activation (9) has also been difficult to amalgamate

with immunological synapse augmented TCR signalling hypothesises. CD28 is believed to be recruited to the cSMAC (2), and as such supports the theory of co-operation with TCR signalling. While it is argued that CD28 may be redundant in initial T cell activation it has a clear role in augmenting and sustaining TCR signalling and may facilitate activation when antigen is limiting (10). CD28 signalling activates PI3K (Phosphatidylinositol 3-hydroykinase) (10, 11) also believed to be stimulated by TCR, but the relative roles of the two pathways remains controversial. PI3K catalyses transfer of ATP to the inositol ring of PIP, PIP2 and PIP3 (3). Generation of PIP2 may contribute to TCR signalling by providing substrate for PLCγ; however the generation of hydroxylated phosphatidylinositol products also recruit 3-phosphoinositide-dependent kinases (PDKs). PDK activity generates functional PKB (protein kinase B) which inactivates glycogen synthase kinase-3 (GSK-3) (3). In its unphosphorylated form, GSK-3 inhibits NFAT nuclear activity by inhibitory phosphorylation (12). Stimulation of CD28 and subsequent inactivation of GSK-3 would sustain the presence of nuclear NFAT promoting TCR signalling.

It is generally believed that the combination of TCR and CD28 signalling starts entry of the resting T cell into G1 phase of cell cycle, inducing expression of cyclins, production of the T cell mitogen interleukin-2 (IL-2) and surface expression of the high affinity α chain of the IL-2 receptor (IL-2R) (13). Reception of IL-2R signalling drives the T cell through cell cycle allowing clonal expansion. Whether this process still requires antigenic stimulation is unclear, but it has been suggested that T cell division can continue after initial stimulation in the absence of antigen. This may be

directly related to enhanced degradation of the cyclin-cyclin dependent inhibitor $p27^{kip1}$ mediated by CD28 signalling and PI3K activity (3).

Successful initiation of T cell activation induces expression of CD40L (14). Binding to CD40 on the APC produces a two way signal between the cells and enhances B7 expression by the APC, inducing further CD28 signalling (15). Establishment of T cell/APC dialogue also induces expression of other costimulatory molecules. Although some of these molecules are reported to sustain clonal expansion to some degree (such as ICOS), their main role seems to be in development of effector cell phenotypes, and direction of target cell function (discussed in section 1.2).

1.1.3 The activated $CD4^+$ T cell

In brief, TCR signalling and costimulation induces expression of new genes. In lymphoid tissue, dialogue between T cell and APC is established by up-regulation of additional surface receptors that contribute to the development of effector function. T cell stimulation is followed by clonal expansion, increasing the number of pathogen specific T cells. Expression of chemokine and adhesion molecules is altered to allow migration of the primed T cell to either B cell areas, promoting antibody production, or from the lymphoid tissue to the site of infection, directing cell mediated responses.

1.2 CD4$^+$ T cell effector function – Th function

Priming of naïve CD4$^+$ T cells generates an activated T cell secreting large amounts of IL-2, promoting clonal expansion. These activated but as yet undifferentiated T cells (Th0) are subject to programmed development into T helper (Th) subsets that mediate appropriate direction of immune response (Figure 1.4). This is associated with the secretion of a variety of cytokines by particular subsets, influencing an array of other immune system cells (Table 1.1). Th effector functions can fall into two main categories, Th1 or Th2 (16). Th responses are generally associated with activation of either cell mediated (Th1 predominant) or humoral (Th2 predominant) responses (17, 18). This simplistic view allows classification of some diseases based upon the Th response required for resolution. However, care should be taken not to over-simplify the Th1/Th2 paradigm, as the distinction between Th responses is often blurred, requiring both cell mediated and antibody responses.

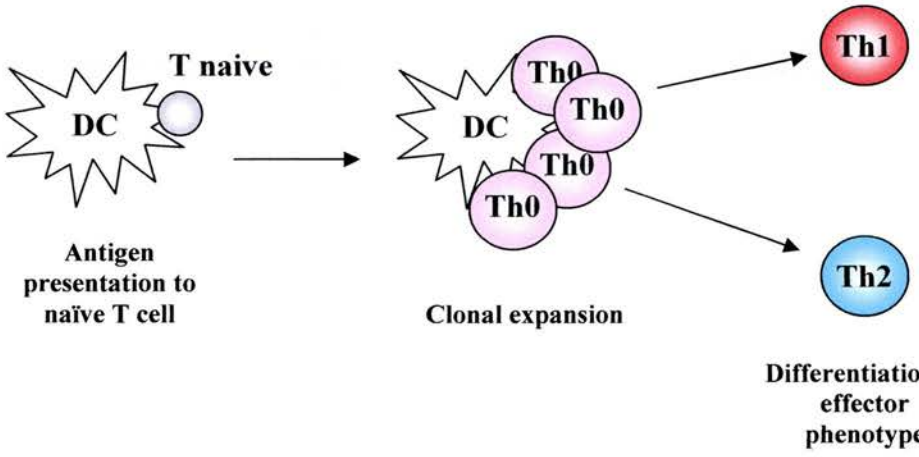


Figure 1.4: Clonal expansion is followed by differentiation. Naïve CD4$^+$ T cells are activated in the draining lymphoid tissue by mature dendritic cells (DCs). Activation induces proliferation of an as yet undifferentiated population of T cells. External stimuli then promote acquisition of a particular effector cell phenotype.

Division of cells into Th1 and Th2 subsets does, however, give a more accurate description of two distinct cytokines profiles that can be generated following $CD4^+$ T cell activation. An individual Th cell can be differentiated to produce either IFNγ or IL-4, being Th1 or Th2 respectively (16). Although a particular cell may be either Th1 or Th2, a polarised clonal/polyclonal population will be generated when one or other phenotype predominates. Factors influencing differentiation of Th1 versus Th2 cells include responses of the innate immune system (19); the cytokine milieu at the time of priming (20, 21); costimulation provided by APCs (22) and the strength of TCR signalling induced (21). Yet the contribution and relative physiological importance of these mechanisms remain controversial.

An additional effector function mediated by $CD4^+$ T cells is the regulation of the immune system. This can occur through cross-regulation between Th1 and Th2 (23), but also occurs through active regulation/suppression. Populations of $CD4^+$ regulatory T cells (T regs) are important in the maintenance of peripheral tolerance, both to self and non-pathogenic antigens (24, 25). Development of this effector function depends on the nature of the regulatory population in question, but can be either predestined or induced upon priming.

Table 1.1: Cytokines associated with Th effector cells
(Cope with cytokines: http://www.copewithcytokines.de/)

Cytokine	Source	Effect
IL-2	Th0, Th1	T cell mitogen
IL-3	Th1, Th2	Progenitor growth factor
IL-4	Th2	Growth, B cell activation, class switching, macrophage activation
IL-5	Th2	Eosinophil differentiation
IL-6	Activated T cell	Growth factor, acute phase response
IL-9	Th2	Mast cell infiltration and proliferation
IL-10	Th2, T reg	Inhibition of Th1, macrophages
IL-12	APC	Secreted by APC, drives Th1 differentiation
IL-13	Th2	Similar to IL-4, alternative macrophage activation
IFNγ	Th1	Inhibits Th2, macrophage activation, NK cell growth
TNFβ	Th1	Macrophage and neutrophils activation, B cell inhibition
TNFα	Th1, some Th2	Local inflammation, acute phase response

1.2.1 Effector functions of Th1/Th2

Th1 and Th2 effector functions allow for the induction of a specific immune response appropriate to the antigen encountered. Classically, Th1 cells are associated with the activation of cell mediated immunity, promoting CTL (cytotoxic T cell) and macrophage activity, Th2 cells function to promote humoral immunity. However, it is clear that this is an over simplification and that elements of Th1 responses require antibody and that removal of extra-cellular pathogens is affected by macrophage function. The following paragraphs will give an indication of the main effects mediated by Th activity and where appropriate indicate where both Th1 and Th2 functions may overlap.

Th1 effector functions can act to promote CTL activity (26), enhance macrophage activation and support production of antibody. These functions are elicited by both secreted cytokines (characteristically IFNγ, IL-2 and TNFβ) and cell/cell interactions. Antigen presentation to $CD4^+$ T cells not only activates the T cell but can influence the function of the cell presenting antigen. $CD4^+$ T cell activation induces expression of the TNF family costimulator CD40L (27). Binding to CD40 expressed by DCs promotes the expression of CD80 and CD86 by the APC (28), enhancing provision of costimulation. This Th function also serves to enhance survival DC survival. Additionally, the contribution of enhanced PKB activity following activation of DC expressed RANK by TRANCE (29) and likely other as yet unidentified interactions serve to "licence" the DC for activation of naïve $CD8^+$ T cells. It is generally regarded that activated $CD8^+$ T cells are poor producers of IL-2, necessary for their clonal expansion and survival. This T cell mitogen is produced in large quantities by activated $CD4^+$ T cells, particularly Th1 cells. In this fashion, Th1 function also supports the proliferation and activity of $CD8^+$ T cells 26.

Th1 cell activation of macrophages is also of critical importance in the elimination of some extracellular and intracellular pathogens (13). Normal macrophage activity is usually sufficient for the destruction of many intracellular or phagocytosed extracellular pathogens. However, some pathogens have developed potent evasion strategies inhibiting normal macrophage functions. Binding of Th1 cells recognising specific peptide/MHC class II complexes on macrophages allows CD40L/CD40 interactions, enhancing macrophage expression of IFNγ receptors (30). Synergistic

effects of IFNγ and TNFβ released by the Th1 cell enhances production of NO (nitric oxide) (30), a potent antimicrobial. IFNγ stimulation of the macrophage also enhances expression of inflammatory cytokines (13) (TNFα, IL-1, IL-6, IL-12), MHC class I and II (31), costimulatory molecules (CD80/86, CD40L) and promotes respiratory burst (13).

It must be stressed however that macrophages can also be activated by Th2 cells (30). Th2 cells secrete both IL-4 and IL-13 which are able to enhance expression of MHC class II and mannose receptors by macrophages (30). IL-4 and IL-13 do moderately reduce respiratory burst and inflammatory cytokine synthesis, but this is not comparable to inhibition by IL-10 (30) (although IL-10 has been classed as a Th2 cytokine its function does appear to be more related to immunoregulation, and will be discussed in this context in the following T reg section, Chapter 1.2.3, page 39). The key difference in the activation of macrophages by Th1 and Th2 cells is in arginine metabolism, which is higher following stimulation by Th2 cytokines (32). The result of this is reduced synthesis and release of NO (32). Th2 activation of macrophages in this fashion may serve to limit damage mediated by prolonged Th1 responses, and as such may also be important in mediating repair processes.

Humoral immunity can be influenced by Th1 cells but is typically associated with Th2 cells. Development of T-dependent-antibody production relies on the recognition of linked antigens by the $CD4^+$ T cell and B cell (33). B cells sample specific antigen and are activated through the B cell receptor complex (34). This primary activation event requires a second, accessory signal provided by the T helper

cell in the form of CD40L/CD40 and IL-4, to drive germinal centre formation, class switching and differentiation into plasma or memory cells (35, 36). The switch to a particular isotype is directed by cytokine signalling, where IL-4 drives murine B cells to secrete IgG1 and IgE (13). Class switching is also induced by Th1 cells with IFNγ driving expression of IgG2a in mice (13).

Th2 cells also secrete IL-5, and IL-9. These cytokines are associated with the activity of two major cell types associated with Th2 responses, eosinophils and mast cells. IL-5 acts directly on bone marrow precursor cells, enhancing their proliferation, driving differentiation into eosinophils and prolongs the viability of terminally differentiated cells (37). Mast cells are also central to Th2 responses, with mastocytosis induction following IL-9 secretion by Th2 cells (37).

1.2.2 Commitment to Th1/Th2 cell fates

The presence of IL-12 or IL-4 at the priming of naïve $CD4^+$ T cell is associated with Th1 or Th2 differentiation respectively. These observations lead to the idea that Th1/Th2 cell fate decisions was an instructive event. It is now clear that numerous mechanisms can influence Th polarisation, likely to be driven by the innate immune system and the availability of antigen.

The existence of individual Th1 and Th2 progenitor cells seems unlikely given the plasticity of $CD4^+$ T cells primed under polarising conditions. Changing the differentiation stimuli could divert polarisation from one Th subset to the other (38, 39). The implications arising from these reports are that primed $CD4^+$ T cells can be

influenced to become one or other Th subset, and that this eventually becomes a stable cell fate as the effect is not seen subsequent to four or five rounds of division. So it appears that a naïve $CD4^+$ T cell has the capacity to develop either Th1 or Th2 characteristics.

Based on the available literature, a likely explanation of how polarisation of Th subsets occurs would encompass variations in costimulatory molecule expression by DCs, the cytokines present during priming and clonal expansion, the biasing of transcription factor expression over successive cell divisions and eventual epigenetic alterations of cytokine loci.

A model of selective differentiation has been proposed, relating a balance of transcription factor expression by the primed T cell. T-bet (a T-box transcription factor) is induced upon activation as is GATA-3 (a zinc-finger transcription factor) (21). Direction of expression in favour of T-bet or GATA-3 results in differentiation of Th1 or Th2 respectively (21, 39). IL-12 signalling is known to drive Th1 cell differentiation, possibly by direct action on T-bet expression. T-bet activity augments sensitivity of $CD4^+$ T cells to IL-12 and remodels the IFNγ locus allowing STAT4, NFκB and NFAT driven transcription (21). Reception of IL-4 during priming promotes GATA-3 expression, which remodels the chromatin structure around the IL-4 gene promoting transactivation by the Th2 specific transcription factor c-MAF (40). GATA-3 also enhances its own expression and that of IL-5 (21).

The factors influencing the biasing of T-bet/GATA-3 expression are likely to be driven by the innate immune response responding to a pathogen. Inflammatory mediators and direct recognition of the pathogen by DCs will influence the context in which antigen will be presented to the naïve $CD4^+$ T cell. IL-12 production by DCs following ligation of particular Toll receptors (19) likely selects for T-bet expression in $CD4^+$ T cells, driving IFNγ production and further Th1 lineage commitment. Early sources of IL-4 are somewhat more controversial, but it may be released following NK T cell activation (20). As discussed earlier, this cytokine selects for GATA-3 expressing cells. It has been proposed that certain costimulatory molecules can influence differentiation of Th1 versus Th2. CD86 induced signalling has been shown to promote Th2 development in some instances, while CD80 promotes Th1 (41). ICOS ligation is also believed to promote Th2 differentiation (41). These may not reflect specific effects of a particular molecule but likely pertain to the overall degree of signalling induced in the naïve T cell.

Differences in signal transduction between Th1 and Th2 cells have been reported and possibly relate to observations that low amounts of antigen or low affinity peptides tend to induce Th2 responses (21). Limiting antigen or low affinity interactions between MHC class II and peptide (resulting in poorer surface stability of MHC/peptide complexes) would reduce available TCR ligand. Low affinity interactions between peptide and TCR would also reduce the degree of TCR signalling. Ultimately in these cases, the reduced TCR signal could be translated into Th2 differentiation. This correlates with reduced accumulation of ZAP-70 in Th2 cells (42, 43). However, this may be induction of a qualitatively rather than

quantitatively different signal. Recently described adaptor proteins such as SAP (SLAM associated protein) and SLAT (SWAT-70 like adaptor molecule of T cells) have been implicated in recruitment of Th1 or Th2 specific signalling complexes (signalosomes) respectively (43). However, the time scale in which accumulation of one "signalosome" at the expense of another occurs would suggest this system functions to maintain Th1 or Th2 differentiation on further TCR stimulation.

1.2.3 Regulatory $CD4^+$ T cells

The existence of regulatory T cells (T regs) was initially proposed before the characterisation of Th1 and Th2 populations. Such regulatory cells proved difficult to clone and the recognition of the antagonistic effects of Th1 and Th2 resulted in the theory of active regulation being met with considerable scepticism. The increased use of animal models of autoimmunity combined with techniques such as T cell depletion and reconstitution has resurrected and confirmed the critical function of this T cell subset in mediating active regulation.

Early evidence supporting the existence of T regs stems back to early studies by Sakakura (44, 45). Thymectomy three days after birth (day 3Tx) rendered female mice infertile secondary to development of oophritis. This pathology was not observed when thymectomy had been carried out after one or seven days of life. After demonstrating that grafted normal thymic tissue restored tolerance, while new-born spleen, adult d3Tx donor spleen or bone marrow injection could not, it was proposed that thymus derived lymphocytes prevented disease. Indeed, thymocytes from normal or d7Tx adults sustained tolerance in d3Tx recipients. Generation of the

suppressor lineage was seen in the thymus but cells were not peripheralised before 3 days post birth.

Extensive characterisation of numerous models of autoimmunity (46) have resulted in the general acceptance that such a regulatory population exists but has raised many questions as to the generation of T regs and how they mediate their effector function. The generation of $CD4^+CD25^+CTLA\text{-}4^+GITR^+FoxP3^+$ naturally occurring T regs in the thymus suggests that they are selected for their ability to respond to self antigens. Thymic generation is thought to occur through a process of "altered negative selection" where interactions with self not strong enough to induce cell death results in T reg differentiation (47, 48). This hypothesis is based on findings that strong antigenic signals are required to induce this regulatory population.

How natural T regs mediate suppression has proved to be a topic of hot debate. *In vitro*, the main mechanism appears to be cell-cell contact dependent; however, *in vivo* models support varying degrees of cytokine contribution (TGFβ and/or IL-10). In this context natural T regs may serve to contain pathology caused by chronic infections, with secreted immunoregulatory cytokines having bystander effects. This could prove to be an efficient method to prevent autoimmunity following exposure to self in the context of pathogen derived "danger signals".

Although natural T regs can prevent autoimmune responses, tolerance to innocuous non-self antigens is also required to prevent inappropriate immune system activation.

As such it has been proposed that populations of T regs can be generated from mature $CD4^+$ T cells exposed to peripheral antigen, of which two populations have been characterised to date, Tr1 and Th3 cells (24, 49). The immunological context in which antigen is detected by a T cell is known to affect differentiation of Th1 and Th2 cells. A similar theory is proposed for generation of regulatory T cells other than the natural $CD4^+CD25^+$ cells. Administration of antigen via intranasal or oral routes, low level TCR stimulation or stimulation in the absence of costimulation may represent contributing factors to peripheral T reg cell generation (46, 24). Repeated i*n vitro* antigenic stimulation of T cells in the context of IL-10 gives rise to such a T reg subset termed Tr1 cells (50). These are high producers of IL-10 themselves, which appears to be the predominant mechanism of regulation. IL-10 displays strong immunosuppressive effects on pro-inflammatory cytokine release by neutrophils, monocytes, macrophages, eosinophils and T cells. *In vitro* derived Tr1 cells are capable of preventing pathology *in vivo* and this was demonstrated in both a colitis and an airway hypersensitivity model (50, 51). High IL-10 titres associated with reduced clinical symptoms following specific immunotherapy, and may represent *in vivo* generation of Tr1 cells (52).

A second population of peripherally generated T regs, Th3 cells, are high producers of TGFβ. Low dose myelin basic protein feeding in a TCR transgenic EAE model lead to the generation of a Th3 population (53). Addition of TGFβ or IL-10 neutralising antibodies revealed an important role for both these cytokines in disease prevention. TGFβ production is vitally important in maintaining tolerance,

demonstrated as a lymphoproliferative disorder in TGFβ1 deficient mice (54), similar to lpr and gld mice.

In reality, the distinction between highly polarised Th subsets is blurred and this likely seems true for T reg subsets. It has been suggested that natural T reg activity can induce peripherally derived T regs during ongoing inflammatory responses, important in preventing pathology during chronic disease. What is clear is that T reg cells do exist as a heterogeneous population that can mediate active regulation via both cell-cell contact and cytokine production in a response appropriate to the immunological stimulus.

1.2.4 Cell fate, Notch and T cells

The decision to respond to antigen and development of a particular effector function are both critical cell fate choices in the generation of an appropriate immune response. Although the developmental signalling pathway, Notch, is classically associated with embryonic cell fate determination it is also known to influence T cell lineage development. This has lead to speculation that if Notch is expressed by mature $CD4^+$ T cells, signalling may influence activation and development of effector function.

1.3 Notch signalling

The Notch signalling pathway controls a broad spectrum of cell fate decisions that occur during multicellular eukaryotic development. The Notch receptor was initially identified in *Drosophila melangaster* (55). *Drosophila* Notch has two ligands, Serrate and Delta. Related proteins of this highly conserved developmental pathway have been identified in numerous organisms ranging from the nematode, *C. elegans*, to humans (mammalian homologues Notch1-4, the Serrate homologues Jagged1/2, and Delta homologues Delta-like 1, 3 and 4 have been documented) (Table 1.2) (55, 56).

Two types of Notch signalling process have been proposed, lateral induction and lateral specification/inhibition (Figure 1.5). In lateral induction, signalling events are occurring between non-equivalent cells (57), where one cell expresses the receptor and the other bears ligand. This mode of signalling permits establishment of distinct cell boundaries. In the case of the developing *Drosophila* wing margin, both dorsal and ventral compartments express Notch whereas Delta and Serrate expression is confined to ventral and dorsal regions respectively. Induction of Fringe expression (a regulator of Notch signalling) by dorsal cells results in reception of Delta induced signals enhancing dorsal expression of Serrate. This potentiates ventral cell reception of Serrate induced signals, generating positive feed-back and maintenance of the cell boundary (57).

Lateral inhibition occurs between equivalent cells, expressing both ligand and receptor. Acquisition of a bias in this expression (possibly due to stochastic events) between cells is amplified until one cell type expresses ligand and the other receptor. The resulting environment resembles lateral induction and the cell receiving the Notch signal adopts the secondary cell fate. The classical example of this is observed in *Drosophila* neurogenesis (57). Within the proneural cluster, all cells express the bHLH transcription factor Achaete/Scute (Ac/Sc), Notch and Delta. Stochastic increase in Delta expression by one cell induces Notch signalling in neighbouring cells and E(spl) mediated inhibition of Ac/Sc activity, resulting in the adoption of the secondary cell fate, development as epidermal cells. This decrease in Ac/Sc activity reduces Delta expression by these cells. The primary cell fate, development as a neuronal cell, follows enhanced Ac/Sc activity due to lack of Notch signalling (57).

Table 1.2: Notch pathway homologues
(Locuslink: http://www.ncbi.nlm.nih.gov/LocusLink/index.html)

Notch receptors		Ligands		Signalling mediator	Target genes
Drosophila	Notch	Delta	Serrate	Su(H)	E(spl)
Human	Notch 1-4	Delta-like 1, 3, 4	Jagged 1, 2	CBF-1/RBP-Jκ	HES and HERP family genes
Mouse	Notch 1-4	Delta-like 1, 3, 4	Jagged 1, 2	CBF-1/RBP-Jκ	
Xenopus	Xotch	X-Delta 1, 2		XSu(H) 1-2	
C. elegans	Lin-12 & Glp-1	Apx-1	Lag-2	Lag-1	

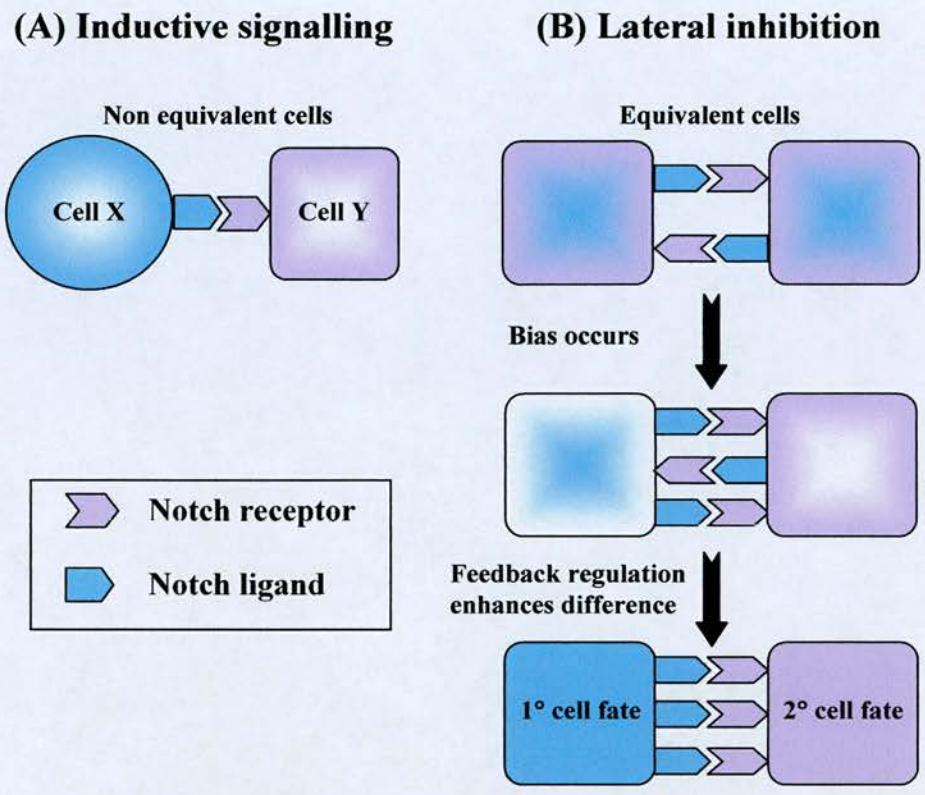


Figure 1.5: Cell fate determination by Notch signalling. (A) Inductive signalling occurs between two non-equivalent cells, one expressing receptor and the other ligand. Cell fate decision of cell Y is determined by the ligand bearing cell X. Signalling in this context maintains defined boundaries between two different cells types. (B) Lateral inhibition occurs between initially equivalent progenitors. Stochastic events or asymmetric cell division can lead to differences in Notch pathway regulator concentrations in progeny resulting in biased expression of receptors and ligands. Feedback signalling maintains and enhances this difference resulting in adoption of primary cell fate by the ligand bearing cell and development of the secondary cell fate by the Notch receptor expressing cell.

1.3.1 The mammalian Notch signalling pathway (Figure 1.6)

The mammalian Notch family consists of four receptors, Notch1, 2, 3 and 4 (56). The mature receptor is generated from a single polypeptide cleaved (S1 cleavage event) by a furin-like convertase in the trans-Golgi (58), producing the functional heterodimeric single-pass transmembrane receptor. Activity of the Fringe family of glycosyltransferases (mammalian Lunatic, Manic and Radical fringe) is also

necessary in the production of a mature receptor (59, 60). Addition of O-linked glucose/fucose to the extracellular domain is vital in mediating ligand binding (59). The extracellular domain consists of multiple epidermal growth factor (EGF)-like repeats (29-36 repeats, depending upon the receptor homologue) (61) and three Lin12/Notch/Glp-1 (LNG) domains (Figure 1.7). Binding of the ligand DSL (Delta/Serrate/Lag) domain to Notch EGF repeats induces signal transduction (Figure 1.6) by allowing ADAM metalloprotease-mediated release of the Notch extracellular domain (S2 cleavage) (62). Subsequent γ-secretase cleavage (S3 cleavage) (63, 64) of the remaining Notch receptor releases the intracellular domain (NICD), allowing nuclear translocation.

The γ-secretase complex is generated from presenilin, nicastrin, Aph1 and pen-2 (65). The exact role played by each component is unclear; however, each is required to produce a functional complex. Mutations in presenilin result in developmental phenotypes similar to those observed in the absence of Notch, and as such presenilin has been suggested to constitute the catalytic subunit (66, 67). A proposed mechanism for generation of functional γ-secretase involves stabilisation of presenilin by Aph-1 and nicastrin (68). Generation of γ-secretase activity requires endoproteolysis of presenilin favoured by pen-2 expression (68). The two resulting presenilin subunits are proposed to constitute the catalytic region of the γ-secretase complex (69). Nicastrin has been shown to bind to Notch (70) and in this context is thought to facilitate binding of the γ-secretase complex to the receptor and guide the catalytic domain to the Notch transmembrane region.

Nuclear translocation of NICD is mediated by two nuclear translocation sequences either side of the RAM23 domain. Once inside the nucleus, NICD binds CBF-1/RBP-Jκ via the RAM23 domain and ankyrin repeats. Binding of NICD converts CBF-1 from a transcriptional repressor to an activator by displacing histone deacetylases and recruiting histone acetylases along with co-activators such as Mastermind-like1 (71), p300 (72) and the adaptor protein SKIP (73). Typically, this pathway activates transcription of the basic helix-loop-helix (bHLH) proteins hairy-enhancer of split family (HES and HERP) (74).

Notch can also signal independently of CBF-1 via a less well defined pathway involving the zinc-finger containing cytoplasmic protein Deltex (75). The role of this protein in augmenting or inhibiting Notch signalling remain to be clarified, but what is known is that Deltex is able to bind Grb2 and modulate Ras and consequently c-Jun N-terminal kinase (JNK) activity (76, 77).

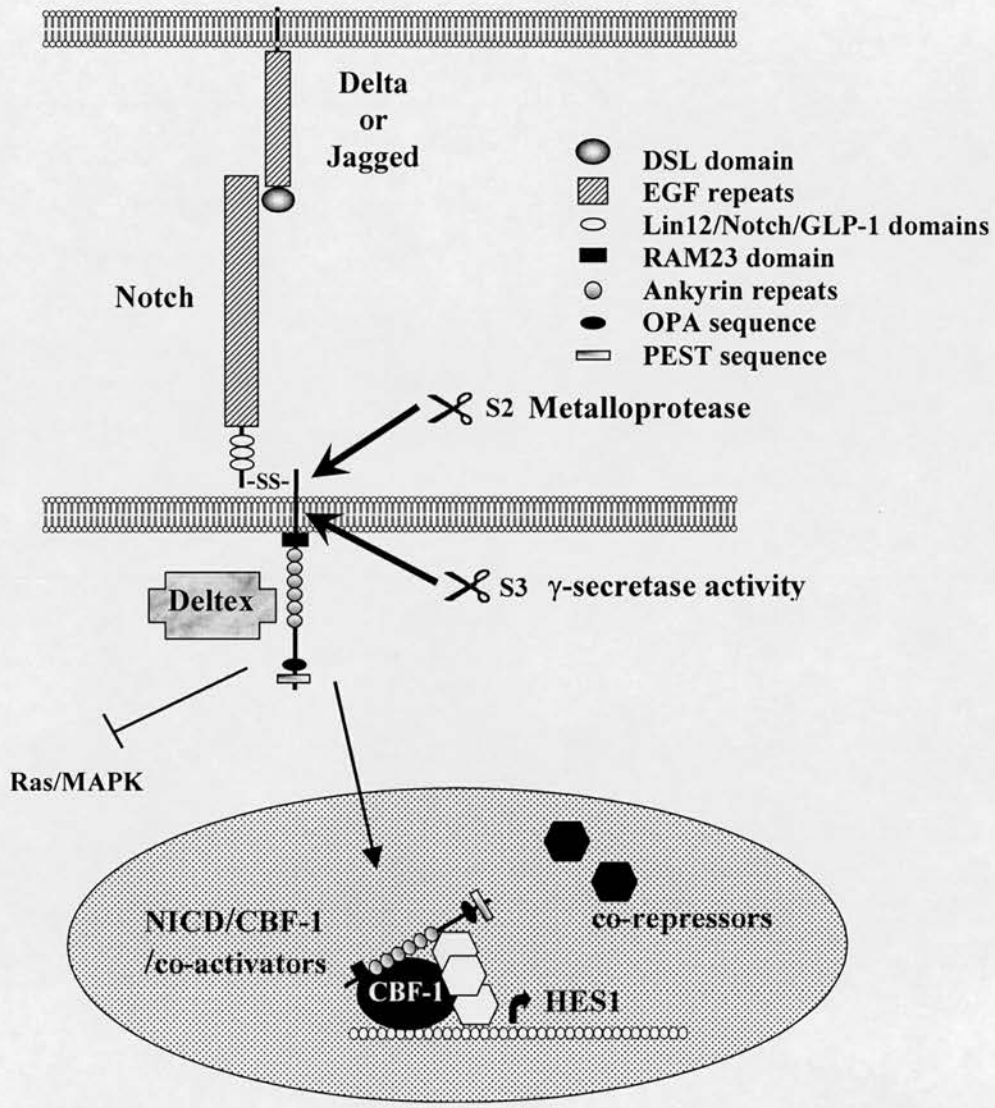


Figure 1.6: Notch signal transduction. Binding of ligand by Notch allows receptor cleavage by a metalloprotease (possibly TACE). The intra-membrane region of the receptor is then cleaved by the γ-secretase complex, releasing the Notch intra-cellular domain (NICD). NICD translocates to the nucleus where it binds to CBF-1 and displaces co-repressors and recruits co-activators inducing transcription of *hes1*. Signalling by Notch through the Deltex pathway is little understood but does inhibit the Ras/MAPK pathway through an interaction with Grb-2.

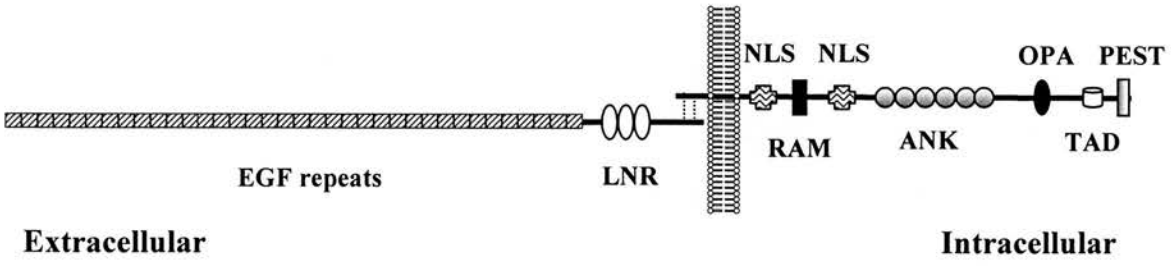


Figure 1.7: Structure of the Notch1 receptor. EGF repeats, epidermal growth factor repeats; LNR, Lin12/Notch/GLP1 repeats; NLS, nuclear localisation sequence; RAM domain, RBP-J association molecule; ANK, Ankyrin repeats; OPA, proline rich domain; TAD, transcriptional activation domain; PEST, proline-glutamate-serine-threonine rich domain.

1.4 Notch and T cell development

Aberrant Notch signalling was linked to dysregulated T cell development in a subset of human T cell leukaemia (78). Since this initial observation a complex interplay between Notch signalling and other signal transduction pathways has been proposed to regulate cell fate decisions in the generation of mature T cells from haematopoietic progenitor cells. Four mammalian Notch homologues have been identified (Notch1-4), the expression of which fluctuates through different stages of thymocyte differentiation (79, 80). Correlation with expression of the Notch target gene *hes* was also observed throughout T cell development (81), indicative of Notch signal transduction.

1.4.1 Notch signalling in thymocytes

Over-expression and abrogation of Notch signalling has emphasised the critical role of this pathway in T lineage commitment (both thymic dependent and independent) (Figure 1.8). Induction of Notch signalling in bone marrow progenitors leads to abnormal T cell development at the expense of B cell generation (82). Conversely, inhibition of Notch signalling supports thymic differentiation of B cells (83, 84). In addition to deletion of Notch, over-expression of molecules known to attenuate Notch signalling prevents T cell but promotes B cell development (85). Inhibition of E2A transcription factors (bHLH transcription factors required in the earliest stages of B cell development) by Notch signalling is the most likely mechanism by T cell development is favoured (86).

Notch signalling also promotes the development of αβ TCR expressing cells rather than γδ TCR. Over-expression of Notch1 signalling in thymocytes drives expression of αβ TCRs even in γδ T cells and β loci disrupted mice (87). This was further highlighted during reconstitution of mice with mixed Notch1+/+ and Notch1+/- bone marrow stem cells (87). In addition to implicating Notch1 as favouring αβ versus γδ development, the observation that this only occurred in the presence of the Notch+/+ cells suggested that γδ/αβ commitment may occur via a process similar to lateral inhibition.

Notch3 transgenic mice develop aggressive T cell neoplasias (88). Development of tumours was associated with mature T cells expressing pre-Tα transcripts (89), not normally associated with this cell type. The surrogate α chain, pre-Tα, is central to expression of the pre-TCR complex at the cell surface (13). Signalling through this complex also drives the selection of successful β chain expression, induce proliferation and differentiation into DP thymocytes (13). Although not fully investigated, this may also support generation of αβ TCR cells at the expense of γδ cells by selection for β locus rearrangements. Further to this observation, pre-Tα has been identified as a direct target of Notch signalling, with the pre-Tα enhancer containing CBF-1 binding sites (90).

The ability of Notch signalling to influence CD8 versus CD4 commitment has been somewhat controversial. Initial studies suggested that Notch signalling favoured development of $CD8^+$ T cells and rather than $CD4^+$ T cells (91). The development of CD8 SP thymocytes could even be induced by Notch signalling in the absence of

MHC class I or class II (91). However, Notch signalling did not support $CD8^+$ T cell development in the absence of both class I and class II MHC molecules, highlighting the importance of TCR signalling in this process. Further investigation of this phenomenon has given rise to contradictory results, with some reports indicating that Notch1 signalling supports both CD4 and CD8 SP differentiation (92) while others demonstrate blockade of either SP phenotype (93). To date this area remains controversial but does highlight another role for Notch in thymocyte development and hints at an interplay with TCR signalling (94).

Study of Notch signalling in thymocyte development has revealed possible functions of Notch that may be relevant to mature T cells. Of particular note was the activation of NFκB in Notch3 transgenic thymocytes (88). This transcription factor is well known as a mediator of inflammatory responses (95), and is activated by TCR signalling (Chapter 1.1). Additionally, Notch signalling in thymocytes has been shown to reduce expression of CD25 and inhibit activity of an NFAT/AP-1 reporter construct (93), the implication being that Notch1 signalling inhibits that mediated by the TCR.

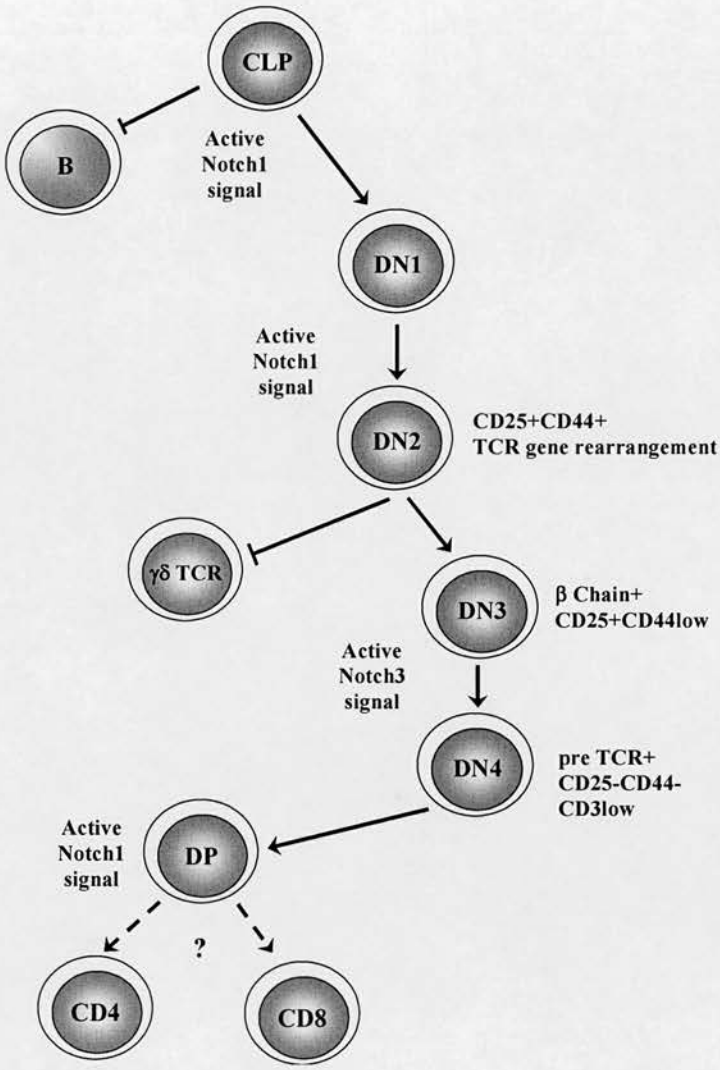


Figure 1.8: Notch signalling and thymocyte development. A simplified over-view of thymocyte development is shown, indicating stages where Notch signal transduction has been shown to promote a particular fate commitment. Thymocyte development is strongly influenced by a combination of pre-TCR/TCR and Notch signalling. Arrows indicate fate decisions favoured by Notch signalling. Common lymphoid progenitors (CLPs) can develop as either B cells or T cells in the bone-marrow or thymus respectively. Development of CD4- CD8- TCR- (DN, double negative) cells is promoted by Notch signalling, resulting in expansion of a DN1 population. At this time the first TCR gene rearrangements are made. Enhanced Notch signalling at this stage increases the number of cells eventually expressing αβ TCRs at the expense of γδ expression. The role that Notch has in determining development of MHC class I or class II restricted T cells is controversial. However, it appears that Notch1 signalling promotes CD8 development in the absence of MHC class I but not CD4 in the absence of class II.

1.5 Notch and $CD4^+$ T cells

The adoption of a particular effector function after recognition of specific antigen is the most crucial fate decision a $CD4^+$ T cell will make. The known processes leading to the stimulation and differentiation of naïve $CD4^+$ T cells are complex and integrated at many levels to ensure the appropriate immune response develops. The Notch signalling pathway can regulate the development of thymocytes through integration with signalling pathways that are also present in mature T cells. Notch receptors and signalling targets are expressed by mature $CD4^+$ T cells and the identification of Notch ligand expression by APCs has generated interest in Notch as a regulator of $CD4^+$ T cell function. Since completion of the laboratory work presented in this thesis, several publications have detailed roles for Notch signalling in T cell function. The findings presented in these publications both complement and conflict with the data presented herein. These aspects will be discussed in subsequent chapters; however, the findings of the aforementioned publications will also be detailed as part of this introduction.

1.5.1 $CD4^+$ T cell expression of Notch signalling components

Gene expression of *notch1* receptor and the Notch ligand *jagged1* (1) can be detected using RT-PCR. Detection of protein has been hampered by the highly conserved nature of this pathway, making generation of high affinity antibodies difficult. Notch1 has been identified in unstimulated spleen and lymph node cells by western blot (96). Activation of these cells with anti-CD3- and anti-CD28-antibodies enhances expression of Notch1 (97, 96). Real-time PT-PCR confirmed this but also

revealed differential expression of the other Notch family receptors by purified $CD4^+$ T cells (97). *Notch1*, *2*, *3* and *4* expression was up-regulated after 16 hours (97).

It has been shown that APCs express Notch ligands and as such it has been assumed that they deliver Notch signals to T cells. Human $CD25^+CD4^+$ T regulatory cells have been shown to express high levels of the Notch ligands *delta-1* and *jagged1* (98) suggesting that $CD4^+$ T cell sub-populations may differentially express components of the Notch signalling pathway. This raises the possibility that some T cells may also be capable of influencing other cell types by delivery of Notch signals.

1.5.2 Notch signalling in activated $CD4^+$ T cells

Expression of the Notch target gene *hes1* is up-regulated upon $CD4^+$ T cell activation, by western blot and real-time RT-PCR (96, 97).The significance of this is unclear but is likely to be related to increased Notch receptor expression and enhanced signal transduction (97).

Inhibitors of γ-secretase activity were initially developed as a therapy for Alzheimer's disease. They have also been demonstrated to be potent inhibitors of Notch signalling (97). Inhibition of the γ-secretase complex prevents release of NICD upon ligand binding, and hence blocks signal transduction. Blockade of Notch signalling in mature $CD4^+$ T cell activation has produced some exciting results although somewhat contradictory to those discussed earlier by Izon et al (99). Reduced $CD4^+$ T cell proliferation was observed in the absence of Notch signalling (96, 97), accompanied by reduced IL-2 secretion (97). IL-2 secretion by anti-CD3-

Ab treated splenocytes from Notch1 anti-sense transgenic mice was found to be significantly reduced but proliferation was unaffected (96). Anti-CD3- and anti-CD28 antibody induction of IL-2 gene transcription is known to involve activation of NFAT, AP-1 and NFκB. Electromobility shift assays (96) have revealed that inhibition of Notch does not affect NFAT activity. However, NFκB activity is dramatically reduced (96), consistent with other findings demonstrating enhanced activity of this transcription factor when T cells are subject to constitutive Notch3 signalling (88) and is decreased in haemopoietic progenitors from Notch1 anti-sense mice (100).

Anti-CD3-Ab induced proliferation in the absence of Notch signalling was not restored on addition of IL-2 to cultures (97). Flow cytometric analysis revealed down-regulation of CD25 expression when Notch signalling was inhibited, suggesting that an inability to respond to IL-2 was responsible for reduced proliferation (97).

Contradictory evidence to the idea that increasing Notch signalling enhances proliferative responses comes from the use of a recombinant Delta1-Fc fusion protein. Delivery of Notch signals using this recombinant ligand did not affect T cell proliferation at doses affecting cytokine production but addition of high concentrations inhibited clonal expansion (101). This may also reflect differential signals derived from different Notch receptors since Delta1-Fc can potentially activate all four Notch receptors. The binding of ligand may also induce a qualitatively different signal in comparison to simply over-expressing truncated

Notch receptors. However, this does not detract from the fact that Notch signalling can influence T cell proliferation in response to TCR stimulation.

Apparent contradictions as to how Notch may affect T cell activation may stem from the differences in TCR signalling strength and consequent masking of any effect Notch may exert on the system. This was demonstrated by inhibition of Notch signalling in DO11.10 TCR Tg mononuclear cells (97). At low dose OVA, cells proliferated less in the absence of Notch. Increasing the concentration of OVA restored proliferation in the absence of Notch. This suggests that Notch may function as a co-stimulatory molecule increasing the sensitivity of TCR signalling, and is consistent with Notch activation of NFκB activity (88, 96, 100). Although no effect on proliferation is seen in the absence of Notch when strong TCR signals are induced, it would be of value to examine other aspects of the system. Notch signalling can rescue DO11.10 hybridoma cells from TCR induced apoptosis by inhibiting activity of the orphan nuclear steroid receptor Nur77 (102). Expression of Nur77 is a feature of activation-induced cell death (103), a key mechanism in the termination of T cell responses (104). TCR/CD3 mediated stimulation can result in proliferation or apoptosis depending on additional signals present at the time of T cell activation (103). Enhanced Notch signalling may promote survival of activated T cells, maintaining immune responses.

1.5.3 Notch and $CD4^+$ T cell effector function

The first publication detailing how Notch signalling could influence T cell function utilised Serrate1 transfected dendritic cells as APCs. Mice were immunised with

either Serrate1 (Jagged1) transfected or control dendritic cells pulsed with the house dust mite antigen Der p1 (1). Antigen presentation in the context of Serrate1 resulted in tolerance, which could be transferred by the $CD4^+$ T cell compartment, suggesting generation of antigen specific regulatory T cells.

Consistent with Notch signalling inducing tolerance, use of allogeneic EBV-lymphoblastoid B cells over-expressing Jagged1 reduced allo-induced proliferation of $CD4^+$ T cells and was associated with reduced levels of IFNγ, IL-2 and IL-5 (most likely due to cell numbers) (105). $CD4^+$ T cells activated in this way produced increased levels of TGFβ. IL-10 secretion appeared unchanged even in the absence of proliferation, perhaps suggesting that more IL-10 was being produced per cell. This cytokine phenotype most likely relates to generation of an antigen specific Th3 regulatory population, capable of transferring hyporesponsiveness to fresh cultures yet not affecting responses to third party antigens (105). These regulatory cells also expressed higher levels of *hes1* transcripts than cells stimulated with normal allogeneic cells, indicating that they had received increased Notch signal transduction.

Mice transgenic for a lck-promoter driven constitutively active Notch3 receptor were protected against induction of experimental autoimmune diabetes (106). Transgenic spleens contained significantly higher levels of $CD25^+CTLA\text{-}4^+CD4^+$ T cells, a phenotype shared by naturally occurring T regulatory cells. The $CD25^+CD4^+$ T cells were also found to express higher amounts of IL-10, at both transcript and protein level (106). Additionally, adoptive transfer of the transgenic $CD4^+$ T cells could

prevent recipient mice developing diabetes. Whether these cells do represent the naturally occurring thymically derived T regulatory cells is uncertain. Stimulation of transgenic spleens also induced elevated levels of IL-4, so perhaps protection against diabetes in this system is due to lack of Th1 polarisation. The data however does strengthen the link between Notch and CD25 expression. It might be speculated that differential Notch signals are induced when cells undergo altered negative selection, a process thought to generate naturally occurring $CD25^{+}CD4^{+}$ T regs. Whether this signal is directly responsible for generating T regs is not clear, however, differential expression of Notch pathway associated genes has been observed in both human and mouse $CD25^{+}CD4^{+}$ T regulatory cells (98, 106).

The use of a recombinant Delta1-Fc fusion protein has been reported to inhibit secretion of pro-inflammatory cytokines by activated $CD4^{+}$ T cells. Delivery of Notch signalling in this context enhances secretion of IL-10, with the resulting polarised population having a cytokine profile reminiscent of Tr1 cells, but it is unclear as to whether these cells have regulatory capacity (107). In a different system, high doses of Delta1-Fc inhibited proliferation of $CD4^{+}$ T cells, this aspect of Delta induced Notch signalling has not been fully explored but seems not to be IL-10 or TGFβ mediated (101). Interestingly, cells activated in the presence of this Delta1-Fc protein developed Th1 like cytokine profiles with high IFNγ secretion (101). Delta1-Fc could even induce $CD4^{+}$ T cell secretion of IFNγ in unstimulated cultures and under Th2 promoting conditions (101). This phenotype was mimicked by over-expression of constitutive Notch3 signalling (101), in direct contrast to a previous report detailing that Notch3 signalling promoted regulatory T cell generation (106).

Hypothesis:

Based on published data relating notch signalling to T cell development it was suggested that Notch signalling may also influence mature T cells. Since $CD4^+$ T play a central role in organisation of immune responses and that this depends on how they differentiate following activation, the following hypothesis was generated:

Notch signalling has a role in peripheral $CD4^+$ T cell differentiation.

The following experimental aims were developed to address this hypothesis. These form the basis of subsequent results chapters and as such give a brief insight into the basis of the work presented herein.

Aims:

Notch pathway expression by $CD4^+$ T cells

- Determine Notch pathway component expression by RT-PCR
- Assess differential expression of these genes following $CD4^+$ T cell stimulation
- Ascertain the possibility of an interaction between the Notch receptor and TCR

Inhibition of Notch signalling in $CD4^+$ T cells

- Develop inhibition protocol
- Assess effects of Notch inhibition on cell viability and proliferation

- Characterise cytokine profile in the absence of Notch signalling
- Address possible transcriptional regulation of T effector phenotype

Characterisation of Notch signalling in regulatory $CD4^+$ T cell populations

- Isolate naturally occurring T regs and generate Tr1 cells
- Demonstrate regulatory capacity of these cells
- Determine expression of Notch components by regulatory $CD4^+$ T cells
- Assess contribution of Notch signalling to active regulation

Induction of Notch signalling in $CD4^+$ T cells

- Over-express Notch ligands on APCs and use to prime naïve $CD4^+$ T cells
- Characterise cytokine profiles of cells in context of ligand induced Notch signalling
- Transfect $CD4^+$ T cells with a constitutively active Notch1 signal
- Characterise proliferative and cytokine responses in the context of Notch1 signalling.

2 Materials and Methods

2.1 Materials

All reagents were purchased from Sigma, Poole, UK, unless otherwise stated.

CFSE (5-(-and 6)-carboxylfluorescein diacetate succinimmidyl ester): CFSE (Molecular Probes, Leiden, Netherlands) was dissolved to a concentration of 5mM in DMSO. Aliquots were prepared and stored at -20°C.

Complete RPMI medium: Murine cells were cultured in RPMI 1640 (Life Technologies, Paisley, U.K.) with 10% FCS (heat inactivated), 100U/ml penicllin/streptomycin (Life Technologies), 2mM L-glutamine plus 50μM β2-mercaptoethanol. Stored 4°C.

Complete ISCOVE'S medium: human cells were cultured in ISCOVES (Life Technology), with 10% FCS (heat inactivated), 100U/ml penicillin/streptomycin (Life Technologies) and 2mM L-glutamine.

DC complete medium: As complete RPMI but without the addition of β2-mercaptoethanol.

DO11.10 NFAT-GFP Hybridoma (108)**:** Kindly gifted by Dr James Brewer (University of Glasgow). The cell line was grown in complete RPMI plus 1mg/ml geneticin. Cells were grown in a 75cm^2 flask, standing upright, in a humidified

incubator at 37°C/5% CO_2. Cultures were split every four to five days depending on cell density.

ELISA Block buffer: PBS containing 1% BSA, 5% sucrose and 0.05% Tween-20, stored at 4°C.

ELISA Reagent diluent: PBS containing 1% BSA (Sigma), stored at 4°C.

ELISA Stop solution: 0.5M H_2SO_4.

ELISA substrate solution: TMB liquid substrate system for ELISA (R&D Systems, Abingdon, UK).

ELISA wash buffer: 0.05% Tween-20 in PBS

FACS wash: 0.1% sodium azide and 0.5% BSA in PBS (without calcium or magnesium). Stored 4°C.

FACS fix: 4% paraformaldehyde in PBS, stored 4°C.

γ-secretase inhibitor: The γ-secretase inhibitor II (MW167) (Merck Biosciences, Darmstadt, Germany) was dissolved in 10mM DMSO to a final inhibitor concentration of 1mM.

GM-CSF supernatant: GM-CSF supernanant was kindly gifted by Dr G. Perona-Wright. Supernant was collected from the X63-gmcsf hydridoma (109). Supernantant contained 200-300ng/ml GM-CSF (by ELISA).

LB medium: Ten capsules of media (Sigma, 1-7275) in 500ml distilled water; autoclaved. Ampicillin or kanamycin at 50μg/ml if required.

LB agar: Ten agar capsules in distilled water; autoclaved. Ampicillin or kanamycin at 50μg/ml where required.

LPS: Escherichia coli lipopolysaccharide serotype 055:B5 powder (Sigma) reconstituted to 1mg/ml in PBS (without calcium or magnesium).

MACs buffer: 0.5% BSA in PBS (without calcium or magnesium), stored at 4°C.

Mowiol Mounting medium: 2.4g of Mowiol (Merck Biosciences) was added to 6g of glycerol and stired briefly with a pipette. 12ml of deionised water was then added and stirred overnight at room temperature 12ml 0.2M Tris (pH 8.5) and heat to 50°C for 1-2 hours while stirring. Once the Mowiol had dissoled, the solution was then spun at 5000 x g for 15 minutes. DABCO was added to 2.5%. The solution was stored at -20°C.

Ovalbumin peptide (OVAp): OVAp 323-339 was purchased from Albachem (Edinburgh, UK). The peptide was reconstituted in dH_20 to give a 3mM stock.

6x PCR loading buffer: 0.25% weight/volume of bromophenol blue and 40% weight/volume of sucrose in distilled water, stored at 4°C.

Recombinant cytokines: IL-2 (R&D Systems) and IL-10 (BD Pharmingen, Oxford, UK) were reconstituted in 0.1mg/ml BSA in PBS (without calcium and magnesium).

Red cell lysis buffer: 1mM NH_4HCO_3 (ammonium hydrogen carbonate) and 114mM NH_4Cl (ammonium chloride) in distilled water. Stored 4°C.

TAE buffer: 100ml of 10X TAE buffer (Life Technologies) made up to 1 litre with deionised water.

2.2 Methods

2.2.1. Mice

6-8 week old female BALB/c mice were purchased from B & K (Hull, U.K.) and housed in the University of Edinburgh Animal Facility in accordance with Home Office guidelines. RAG-/- DO11.10 TCR Tg mice were maintained as an in house breeding colony, housed in isolators.

2.2.2. Cell purification

Spleens were removed from BALB/c or DO11.10 TCR Tg female mice aged 6-8 weeks. Tissue was ground using 1 ml syringe plunger through a 70μm cell-strainer (Becton Dickinson, Oxford, UK) into complete RPMI. Cells were then pelleted (300g for 7 minutes) and resuspended in 4 ml of cold red cell lysis buffer and left on ice for 2 minutes. Cells were then diluted in complete RPMI and washed 300g for 7 minutes at 4°C. Cells were then resuspended in 10ml complete RPMI for counting.

To remove dead cells, spleen suspensions were resuspended in 2ml of complete RPMI and layered gently onto 2ml of room temperature lympholyte-M (Cedarlane, Ontario, Canada) per spleen. Cells were then spun for 25 minutes at 1500g. The interface was then removed and washed twice in complete RPMI. Cells were then resuspended in 10ml and counted using a haemocytometer.

$CD4^+$ T cells were purified from mouse spleens using MACS CD4 isolation kit by autoMACS (Miltenyi Biotech, Bisley, U.K.) according to manufacturers intructions.

Cell purities were 95±4% by flow cytometry. Briefly, cells were resuspended in MACsbuffer and incubated with biotinylated antibodies against CD8α, CD11b, CD45R, DX5 (anti-NK cell) and Ter-119 (anti-erythrocyte) for 10 minutes at 4°C. Anti-biotin microbeads were then added and incubated for a further 15 minutes. Cells were then washed and resuspended in MACSbuffer for running through the autoMACs (using "Deplete" program).

Purification of murine naturally occurring T regs employed the MACs $CD25^+CD4^+$ purification kit. $CD4^+$ T cells are isolated (using autoMACs program "depl025") then stained with anti-CD25-PE. $CD25^+$ cells were then positively selected using anti-PE magnetic beads. Cell suspensions were passed through the autoMACs using the "possel DS" program. $CD25^+CD4^+$ cells were 89.7±1.6% pure. However, the purity of the $CD25^-CD4^+$ population derived from this protocol was consistently below 70%. To combat this, $CD4^+$ cells were then positively selected from the negative fraction using MACs L3T4 magnetic beads (autoMACs "possel" program), raising the $CD25^-CD4^+$ purity to 91±3%.

Human $CD4^+$ T cells were isolated from single donor Buffy coats, obtained from the Blood Transfusion Service (Royal Infirmary of Edinburgh, UK) after viral screening. Briefy, one Buffy coat was made up to a volume of 200mls with PBS (without calcium and magnesium). 25ml of blood was layered onto 15ml Histopaque 1077, in 50ml Falcon tubes. These were then spun at 800g for 25 minutes at room temperature. The mononuclear cell layer was aspirated and washed with PBS twice (300g for 7 minutes). Cells were then counted using a haemocytometer before red

cell lysis, pelletted cells being resuspended in 4ml RBC lysis buffer per $1x10^8$ cells. Cells were incubated on ice for 2 minutes, then washed twice in PBS. Cells were then resuspended in complete ICOVES and counted using a haemocytometer.

$CD4^+$ T cells were then purified from mononuclear cell suspensions using MACs $CD4^+$ T cell Isolation Kits (Miltenyi Biotech), according to manufacturer's instructions. Labelled cells were isolated using the autoMACs "Deplete" program.

2.2.3. Flow cytometry

Flow cytometric analysis was carried out on a Becton Dickinson FACSCalibur (BD Immunocytometry Systems). $2x10^5$ cells per sample were washed with FACS wash for 7 minutes at 300 x g at 4°C. Cells were then incubated in FACS wash plus 10% normal mouse serum (Scottish Antibody Production Unit – SAPU, Carluke, Scotland) for 15 minutes at 4°C to decrease non-specific binding of labelled antibody. Cells were suspended in 50µl of FACS wash prior to addition of 0.2µg of PE- and/or 0.5µg of FITC-labelled antibody. Cells were incubated at 4°C in the dark for 20 minutes. Suspensions were then washed twice with cold FACS wash, resuspended in 100µl FACS wash combined with 100µl FACS fix. Cells could then be stored at 4°C for up to three days if analysis could not be carried out immediately.

Cell viability was assessed using Annexin V-PE/7AAD apoptosis detection kit (BD Pharmingen). Briefly, cells were washed with cold PBS and resuspended in 1X binding buffer. Cells were then incubated with 5µl of Annexin V-PE and 5µl of 7AAD per $1x10^5$ cells (100µl volume). Cells were incubated for 15 minnutes at room

room temperature in the dark. 400µl of binding buffer was then added and staining analysed immediately by flow cytometry.

Delta1 staining was perfomed using a goat anti-Delta1 (T-20) antibody (Santa Cruz: Insight Biotechnology, Middlesex, UK). $2x10^5$ cells were stained using the unconjugated T-20 antibody at a dilution of 1 in 50, incubated for 30 minutes at 4°C, then washed three times. Cells were then stained with a biotinylated bovine anti-goat-IgG (Santa Cruz) diluted 1 in 100, for 15 minutes at 4°C, washed three times and incubated with streptavidin-PE (Pharmingen) (1 in 400 dilution) for 10 minutes at 4°C in the dark. Cells were washed three times, respspended in 100µl FACS wash/100µl FACS fix.

Table 2.3: Antibodies used for flow cytometry

Anti-	Clone	Isotype	Conjugate	Supplier
CD3ε	145-2C11	HsIgG, gp1, κ	PE	BD Pharmingen
CD4	GK1.5	rIgG2b, κ	FITC/PE	BD Pharmingen
CD11c	HL3	HsIgG, gp1, λ	PE	BD Pharmingen
CD25	3C7	rIgG2a, κ	PE	BD Pharmingen
CD80	16-10A1	polyclonal HsIgG	PE	BD Pharmingen
CD86	GL1	rIgG2a, κ	PE	BD Pharmingen
I-A/I-E	2G9	rIgG2a, κ	FITC	BD Pharmingen
Ly-69	RB6-8C5	rIgG2b, κ	PE	BD Pharmingen
Delta1	N/A	polyclonal goat IgG	N/A	Santa Cruz
Goat IgG	N/A	polyclonal bovine IgG	Biotin	Santa Cruz
Human CD4	MT310	mIgG1κ	PE-Cy5	DAKO

2.2.4. Proliferation assays

For polyclonal stimulation of BALB/c $CD4^+$ T cells, cells were seeded at $2x10^5$ cells per well into a 96-well (round-bottom) plate and were unstimulated or treated with plate bound anti-CD3- (eBioscience, San Diego, CA, USA) at 0.1μg/ml in the absence or presence of anti-CD28-Ab (eBioscience) at 1μg/ml. Cells were incubated at 37°C, 5% CO_2 (humidified). Plates were pulsed with 0.5μCi [^{3}H] TdR (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) 16 hours before harvesting. Plates were harvested and read on a betaplate scintillation counter (Wallac, Milton Keynes, U.K.).

For adenovirus infected DC (Chapter 2.2.12, page 79) activation of $CD4^+$ T cells, DCs were pulsed with 0.3μM OVAp for 2 hours. Pulsed DCs were then washed with complete RPMI and seeded with purified DO11.10 $CD4^+$ T cells at a ratio of $1x10^4$ DC to $5x10^4$ T cells in 96 well plates. Plates were pulsed and harvested as before.

For polyclonal stimulation of human $CD4^+$ T cells, cells were seeded as above. Cells were stimulated with plate bound anti-CD3- (BD Pharmingen) in the absence or presence of soluble anti-CD28-Ab (BD Pharmingen). Plates were pulsed and harvested as before.

For $CD25^+CD4^+$ T cell cultures, $CD4^+CD25^-$ T cells were seeded at $5x10^4$ cells per well of 96 well plates, in ratios of 0:1, 1:2 and 1:1, $CD25^+CD4^+$: $CD4^+CD25^-$. Each well contained $1x10^5$ mitomycin C treated BALB/c splenocytes. Control cultures of 10^5 $CD4^+CD25^-$, the maximum number of T cells used in any well, were included to

ensure reduced proliferation in co-cultures was not due to nutrient depletion. Cells were stimulated using plate bound anti-CD3-Ab (0.1μg/ml) and soluble anti-CD28-Ab (1μg/ml). Plates were pulsed and harvested as above.

Tr1 cell co-cultures were carried-out as for $CD25^{+}CD4^{+}$ T cell cultures, with the exception that live splenocytes pulsed with 0.3μM OVAp (for 2 hours then washed) were used as the activation stimulus.

2.2.5. Cytokine assays

$CD4^{+}$ T cells were seeded and stimulated as for proliferation assays. Secretion of IL-2, TNFα, IFNγ, IL-4 and IL-5 was analysed by Th1/Th2 murine cytometric bead analysis (CBA) (BD Pharmingen) from 24, 48 and 72 hour supernatants using the FACSCalibur flow cytometer and BD CBA software. IL-10 secretion was assessed by duoset ELISA (R&D Systems Europe, Abingdon, U.K.). For ELISA, enhanced protein binding capacity 96-well flat-bottom plates were used (Fisher Scientific, Leicestershire, UK). Plates were read using Biorad's Microplate Reader 450 (Biorad Laboratories, Hemel Hempstead, U.K.).

2.2.6. RNA extraction

Total RNA was extracted from cells using the Qiagen RNeasy Mini kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Cells were resuspended in the appropriate volume of supplied RLT lysis buffer and homogenised using Qiagen's QIAshredder spin columns. 70% ethanol was added to the lysate and transferred to RNAeasy spin columns and centrifuged. Columns were then washed

with supplied RW1 buffer and contaminating DNA was digested using Qiagen RNase-Free DNase according to manufacturer's instructions. Columns were then washed with supplied RPE buffer and RNA was eluted in 30µl RNase/DNase free water.

RNA concentration was determined based on absorbance at 260nm and 280nm measured using a spectrophotometer (Ultraspec 200, Pharmacia Biotech, Framingham, MA, USA). Concentration and a ratio of absorbance at 260 and 280 (for high quality, RNA around 1.8) were calculated by the spectrophotometer.

RNA samples were then checked for DNA contamination by running samples as described for RT-PCRs using the Access RT-PCR kit (Promega, Southampton, U.K.) but without addition of the reverse transcriptase, i.e. run as a PCR, for the house-keeping gene β-actin. Generation of a band of 153bp in the absence of reverse transcription would indicate the presence of genomic DNA.

2.2.7. RT-PCR

Reverse transcription of total RNA into cDNA and its amplification was performed using the Access RT-PCR kit (Promega), according to the manufacturer's intructions. Primers were purchased from MWG Biotech (MWG Biotech AG, Ebersburg, Germany). After optimisation, thermocycler conditions were: 1 cycle at 48°C, 45 minutes; 1 cycle at 94°C, 2 minutes; 35 cycles of [94°C for 30 seconds, 1 minute at T annealing°C; 2 minutes at 68°C] and 1 cycle at 68°C for 7 minutes. The thermocycler used was a Pelter Thermal Cycler (PTC)-200 (MJ Research, Watertown, MA, USA).

Samples were loaded on 2% agarose gels (SeaKem LE agarose, FMC) containing 0.5μg/ml ethidium bromide. Gels were prepared using 1x TAE buffer. 8μl of sample was added to 2μl of 6x PCR loading buffer and loaded into a well. The gel was then run for 1 hour at 80volts in 1x TAE. PCR products were detected using an ultraviolet transilluminator (UVP) in conjunction with UVGrab computer software. PCR product size was determined using PCR marker (50-2000bp ladder).

Table 2.4: RT-PCR primers

Gene	Primer Sequence fp: forward primer rp: reverse primer	PCR product length	T annealing (°C)
notch 1	fp 5` TGTTAATGAGTGCATCTCCAACCC 3`	638bp	58
	rp 5` CATTCGTAGCCATCAATCTTGTCC 3`		
notch 2	fp 5` CAGAGGAATAGCAAGACGTGCAAG 3`	599bp	58
	rp 5` GATGAAGAACAGGATGATGACAACAG 3`		
notch 3	fp 5` AGGATCAGTGCAGTAGAG 3`	526bp	48
	rp 5` AGACTGAAGGTAGAGGAG 3`		
notch 4	fp 5` CTACTGCCCACAAGTAGCTGG 3`	183bp	58
	rp 5` CTCGGAGATAGCAGTGACTGG 3`		
jagged 1	fp 5` AGATATACCGCACCCCTTCAG 3`	289bp	58
	rp 5` GGGGGTCACTGTCAGAATGA 3`		
jagged 2	fp 5` ATCTGCGAGCTGGTGGAT 3`	282bp	58
	rp 5` TATACCAGAGGGTGCGACA 3`		
delta-like 1	fp 5` CTGTGACAAACCAGGGGAGT 3`	458bp	60
	rp 5` CCTCCGTCAGGGTTATCTGA 3`		
hes1	fp 5` AATGGAGAAAAATTCCTCCTCC 3`	350bp	58
	rp 5` TCACCTCGTTCATGCACTCG 3`		
β-actin	fp 5` CCACCAACTGGGACGACATG 3`	153bp	58
	rp 5` GTCTCAAACATGATCTGGGTCATC 3`		

2.2.8. Real-time RT-PCR

A Taqman based system and ABI Prism 7700-sequence detector were used for real-time PCR (Applied Biosystems, Warrington, UK). cDNAs were generated from purified RNA samples using the MultiScribe RT kit (Applied Biosystems) using the following reaction mixture: 1µl 10X Taqman RT buffer; 2.2µl magnesium chloride; 2µl dNTP mix; 0.5µl random hexamers; 0.2µl RNase inhibitor; 0.25µl MultiScribe RT; 3.85µl RNA (400ng) – per sample. The thermocycler (Perkin Elmer) conditions were: 1 cycle at 25°C for 10 minutes; 1 cycle of 48°C for 45 minutes then 1 cycle at 95°C for 5 minutes. cDNAs were then diluted 5:1 in RNase free/Dnase free water (Promega).

The real-time PCR reaction relies on 5` -3` nuclease activity of the ApliTag DNA polymerase used. A 5` fluorescently labelled probe (FAM reporter for gene of interest and VIC reporter for ribosomal 18S internal control) with a 3` fluorescent quencher (TAMRA) anneals to the template between the unlabelled PCR primers. Fluorescence is quenched while the probe is intact. The reporter is released by the DNA polymerase degradation of the probe, resulting in an increase in fluorescent emission. Measurement of amplification in direct proportion to increased fluorescence by the sequence detector (ABI Prism 7700) is continuous. The C_T (cycle threshold: point of recognition of threshold of exponential increase in fluorescence) value is indicative of the quantity of input target (i.e. the higher the concentration of template the lower the C_T value).

Data was analysed using the comparative C_T method (as per manufactures instructions). This generates a relative expression value compared to a calibrator positive control, designated the numerical value of 1. The amount of target, normalised to the r18S endogenous reference and relative to the calibrator is given as $2^{-\Delta\Delta C}{}_T$ where ΔC_T is the C_T of the gene of interest – C_T of r18S (normalisation); and $\Delta\Delta C_T$ is the ΔC_T for the target - ΔC_T for the calibrator. A two fold change in relative gene expression is considered to be biologically relevant (110, 111)

Table 2.5: Real-time RT-PCR primers and probes

Primers	Primer Sequence Forward primer (5`-3`) Reverse primer (5`-3`)	Probe sequence
notch 1	TCCAGAGTGCCACCGATGT TCCACCGGCTCACTCTTCAC	CTGCCTTCCTAGGTGCTCTTGCGTCA
notch 2	ACCCTCCGCCGAGACTCT TCCCAGAACCAATCAGGTTAGC	CCTGTCCCACAGGTTCACGGCG
notch 3	GGCTGCAACACTGAGGAATGT TCAGGAGGCAGAAGAACTGTGA	CCGGGACCTCGCTGGCACA
notch 4	TGTCTCCCCCATAGAGTATGCA CTCGAAATCAACTTTGTCCTCTTG	CCGGACATCCTAAACCCTCTTCCCATTG
delta-like1	TTCTTTCGCGTATGCCTCAA CATCAGGCAGGCTGAAGGA	ACTACCAGGCCAGCGTGTCACCG
jagged 1	CCCGCACCCAGGATGTGT CACCCCAGTTGGTCTCACAGA	CACCTGCAATGAACCCTGGCAGTG
jagged 2	CAGCTGGACGCCAATGAGT GCCAATCAGGTTTTTGCAAGA	AGCATTAAGGCACGGCTTCCCTTCA
hes 1	GCTTCAGCGAGTGCATGAAC TTGATCTGGGTCATGCAGTTG	TGACCCGCTTCCTGTCCACGTG
deltex	GCCTCTAGCCTGGCACATG CCAGATCCCCTTAGCGCTTCT	CTCCCTCCGCTCTCGGCGG
IL-10	CCACAAAGCAGCCTTGCA AGTAAGAGCAGGCAGCATAGCA	AGAGCTCCATCATGCCTGGCTCAGC

2.2.9. Confocal microscopy

Cells were smeared onto Superfrost slides (BDH Laboratory Supplies, Poole, Dorset), air dried, fixed for 10 minutes in 90% dry acetone/ 10% methanol and washed in PBS. Cells were blocked in Dako diluent (DAKO, Ely, U.K.) for 10 minutes, incubated with anti-CD4 Ab (BD Pharmingen), washed, and incubated with goat anti-rat Alexa Fluor 594 (Molecular Probes, Leiden, The Netherlands). The isotype rat IgG2bκ (BD Pharmingen) was used as with the CD4 stain. Notch1 staining was demonstrated using a mouse monoclonal antibody directed against the Notch1 EGF repeats 11 and 12, designated A6, isotype mouse IgG2bκ. Cells were stained with A6-FITC (kindly supplied by Viragen, Watertown, USA), washed, incubated with rabbit anti-FITC (DAKO), washed then stained with goat anti-rabbit Alexa Fluor 488 (Molecular probes). All incubations were performed at room temperature for 30 minutes. Slides were then incubated for 10 minutes with ToPro3 (Molecular Probes) to allow visualization of the nucleus, prior to cover slipping. Images were captured using a Leica TCS NT confocal system and analysed using LCS Lite software (Leica Microsystems, Mannheim, Germany).

2.2.10. Tr1/Th cell line generation

This protocol was based on a 1997 Groux et al publication (50). Tr1 cell lines were derived from $CD4^+$ DO11.10 TCR Tg T cells. Spleens were homogenized, washed and resuspended in 4ml complete RPMI per spleen. 1 ml of spleen cell suspension was added per well of a 24 well plate. 0.5ml of CM was added containing OVA peptide (OVAp, 323-339), IL-2 and IL-10 to final concentrations of 0.3μM, 20ng/ml

and 10ng/ml respectively. Cells were incubated at 37°C, 5% CO_2. Cells were passaged after 7 days: cells were harvested by pipetting, pelletted, resuspended in complete RPMI (0.5ml per well collected). 2ml of cells were layered over 2ml of room temperature lympholyte-M (Cedarlane). Cells were then spun for 25 minutes at 1500g. The interface was then removed and washed twice in complete RPMI. Cells were then resuspended in 10ml and counted using a haemocytometer.

The cells were then seeded at $2x10^5$ per well of a 24 well plate in 1ml complete RPMI. 0.5ml of $5x10^6$ mitomycin C treated BALB/c splenocytes (50μg/ml for 30 minutes at 37°C, then washed three times in complete RPMI) were added as feeder cells. Peptide, IL-2 and IL-10 were added as before. Cells were passaged in this fashion three times before use in experiments. Cells to be used experimentally were harvested 7 days after their last passage and incubated for 7 days in 1.5ml (total $6x10^5$ T cells) with IL-2 at 1μg/ml.

Th cell lines were generated following the same protocol but in the absence of IL-10.

2.2.11. DC enriched population

This protocol was based on the Inaba method for generating bone marrow derived DCs (112). Bone marrow from BALB/c mice was extracted from cleaned the femurs and tibiae (surrounding tissue was removed by rubbing with anti-bacterial disinfectant wipes). Intact bones were rinsed in 70% ethanol and washed with PBS. The bone ends were then cut using a disposable scalpel and the marrow flushed through with DC complete medium (DC CM) using a syringe with a 0.45mm (26-

gauge) diameter needle. Cells were centrifuged for 7 minutes, 300g. The pellet was then resuspended in RBC lysis buffer (1ml per mouse) and incubated on ice for 2 minutes. Cells were then washed in DC CM and resuspended in 10ml for counting.

Cells were seeded at $3.75x10^5$ cells/well of a 24-well plate in 1ml DC CM plus 5% GM-CSF supernatant and incubated at 37°C, 5% CO_2. Culture medium was aspirated of after 3 and 6 days of culture. This involved gently swirling the plate before aspiration so as to remove non-adherent granulocytes and B cells without dislodging loosely adherent clusters of developing DCs. Medium was replaced with fresh DC CM plus 5% GM-CSF supernatant. On day 7, loose DC aggregates were removed by pipetting, washed and counted. Where matured DCs were required, day 7 cells were stimulated with 100ng/ml LPS for 5 hours, washed, then used in culture.

2.2.12. Adenoviral infection of DCs

$3x10^6$ bone marrow derived DCs were plated in 600µl DC CM per well of a 6 well plate. Cells were infected with adenoviral constructs at MOIs (multiplicity of infection) from 0 to 400. DCs were incubated at 37°C, 5% CO_2 for 1 hour. Cells were then washed and reseeded at $3x10^6$ cells in 3ml DC CM plus 5% GM-CSF supernatant per well of a six well plate. Infected DCs were then cultured for a further 24 hours to allow for transgene expression. All adenoviral work was carried out in Class II safety cabinets. All equipment in contact with adenovirus was thoroughly bleached (Mini-Haz tabs, Guest Medical, Kent, UK) and the area treated with germicidal ultraviolet light.

Adenoviral constructs used were the dl70-3 empty control vector (113), Ad-GFP (kindly gifted by Drs R. Marr and F. Graham, Department of Biology, McMaster University, Hamilton, Ontario, Canada) and Ad-delta1 (Dr S. Lindley, Immunobiology Group, Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK). The Ad-delta1 virus was generated as follows. Briefly, cDNA encoding murine *delta-like1* was inserted into pDK6, containing a MCMV promoter. E1 transformed 293 cells were then co-transfected with this construct and pBHG10 (an E1/E3 deleted Ad5 plasmid). The virus resulting from homologous recombination was then isolated through plaque purification.

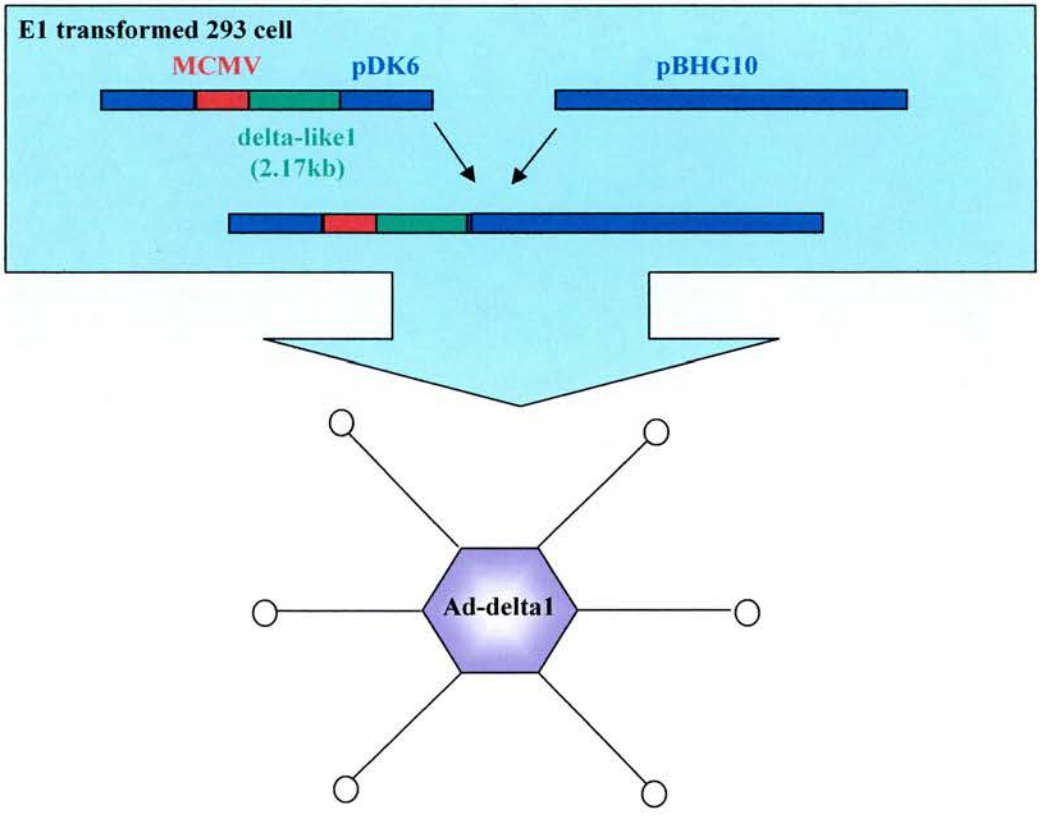


Figure 2.1: Ad-delta1. Ad-delta1 was generated from the homologous recombination of delta-like1 cDNA inserted into the shuttle vector pDK6 and the E1/E3 deleted Ad5 plasmid DNA pBHG10 in HEK293 cells transgenic for adenoviral E1.

2.2.13. Generation of pNICD

pNICD was generated by insertion of the human sequence for a myc-tagged Notch 1 constitutively active intracellular domain into the commercially available pEGFP-N1 vector (BD Biosciences) (Accession number U55762). The human Notch ICD sequence was a kind gift from Professor Raphael Kopan (114) in the form of the pSC2-N-ICv construct (Ampicillin resistant vector).

pEGFP-N1 was digested using Hind III (Promega) and Apa I (Promega) in multicore buffer (Promega) at 37°C for 3 hours. pSC2-N-ICv was also digested using Hind III and Apa I in multicore buffer for 3 hours at 37°C to release the N-ICv sequence with the myc tag. The digests were then run on a 0.8% agarose gel (SeaKem LE agarose, FMC), revealing the linearised pEGFP-N1, the pSC2 construct and N-ICv insert. Product sizes were determined using a 1kb ladder (0.25-10kb, Promega). The linearised pEGFP-N1 and N-ICv were then extracted using the Qiaquick Gel Extraction kit (Qiagen). After extraction, DNA was resuspended in 30µl RNase and DNase free water. pEGFP-N1 and N-ICv were then ligated overnight at 16°C at a ratios of 1:1 and 1:3 (vector:insert). with T4 ligase. Competent cells were then transformed with the ligation mix, then shaken at 37°C for 30 minutes in the presence of kanamycin (50µg/ml). Transformed cells were then plated out and cultured overnight on kanamycin LB plates. Colonies were picked the following day and cultured in kanamycin LB medium for a further 24 hours before extraction of plasmid DNA using the Promega Wizard plus SV Miniprep DNA purification kit. The resulting plasmid preparation was assessed for the presence of the N-ICv insert

by digestion with Hind III and Apa I (3 hours at 37°C). The successfully ligated N-ICv and pEGFP-N1 was designated pNICD.

Competent cells were transformed with pNICD and cultured in 400ml cultures of LB medium/kanamycin overnight. pNICD was then purified using the Qiagen Maxiprep kit. Bacterial pellets were resuspended in RT cell lysis buffer for 5 minutes, then the solution was neutralised. The preparation was then added to QIAfilters, incubated for 10 minutes then filtered by insertion of the plunger.

2.2.14. $CD4^+$ T cell transfection

Purified human $CD4^+$ T cells were transfected using Amaxa nucleofection technology (Human T cell Nucleofector Kit, VPA-1002). Briefly, cells were suspended in supplied transfection buffer and placed into the supplied transfection cuvettes. Cells were either mock transfected or in the presence of 1μg plasmid DNA. Cells were then plated into 96 well plates ($2x10^5$ cells per well) in pre-warmed complete ISCOVE'S.

2.2.15. Statistical analysis

P values were calculated using the statistical analysis function available in Microsoft Excel. A paired T test was carried out to test that the mean difference between paired data in each experiment was not different. P values of less than 0.05 were considered significant.

3 Expression of Notch pathway components by $CD4^+$ T cells

3.1 Introduction/background

APCs have previously been reported as expressing Notch ligands and as such have the potential to deliver Notch signals (115). Additionally, APCs over-expressing Notch-ligands are capable of inducing populations of regulatory T cells (1,105,116). The direct interaction of these ligands with receptors on T cells remains to be established since Notch receptor expression by $CD4^+$ T cells has not been formally demonstrated. Thymocytes express Notch receptors and this pathway has been shown to be vital in development of mature T cells (80,117-119). One particular study implicated Notch in attenuation of TCR signalling where ICN1 (NICD1) over-expressing DP thymocytes exhibited reduced ability to express CD5 and CD69 upon TCR/CD2 co-engagement, proposing that Notch inhibits TCR signalling (99). This effect was further demonstrated in Jurkat cells, Notch activity inhibiting activation of an NFAT/AP-1 reporter construct (99). Extrapolation of this hypothesis to mature T cells presents a mechanism by which Notch signalling may shape T cell responses.

The first step in addressing the overall hypothesis that Notch signalling is able to influence $CD4^+$ T cell effector function was to determine whether or not these cells express components of the Notch pathway (Notch receptors 1, 2, 3, 4; the ligands Jagged1, 2, Delta-like 1; and downstream signalling molecule Hes1). Notch gene

expression is differentially regulated in developmental systems as a means of setting-up boundaries between different cell types (lateral induction). Little evidence presently exists detailing factors regulating *notch1*, *2*, *3* or *4* gene expression. Notch signalling may itself regulate ligand expression, mediated by Hes (57). Further examples of Notch pathway gene regulation are seen in activation of *jagged* and *delta* transcription by NFκB and Ras activity respectively (95, 120, 121). This poses the possibility that Notch genes may be differentially regulated by T cell activation, both Ras and NFκB being targets of TCR/costimulatory signalling (2, 10, 122). Altered expression of these genes may reflect changes in a T cell's requirement for Notch signals and might relate to a particular T cell function.

Molecules capable of influencing TCR signalling such as CD4, CD28 and CTLA-4 are targeted to the immune synapse (2, 123). Localisation with the TCR brings signalling components into close proximity facilitating augmentation or attenuation of signal transduction (2, 124). For Notch to influence TCR signalling, Notch receptors might be expected to localise to the immune synapse. This would also be consistent with the hypothesis that Notch signalling attenuates TCR signalling in thymocytes (99), and place Notch in an ideal situation to influence mature T cell function.

Chapter aims:

- To purify $CD4^+$ T cells from BALB/c spleens;
- Demonstrate activation using anti-CD3- and anti-CD3/28-Ab, measuring proliferation and cytokine production;
- Determine expression of Notch pathway components by RT-PCR;
- Assess differential Notch gene expression between unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells;
- Establish time course for visualisation of T cell polarisation based on induction of CD4 capping;
- Examine surface expression of Notch1 and localisation with an immune synapse-associated molecule (CD4) in resting and activated T cells.

3.2 Gene transcripts for Notch receptors, ligands and downstream signalling components are present in $CD4^+$ T cells

To address whether $CD4^+$ T cells express Notch pathway components, cells were MACs isolated from BALB/c spleens (Chapter 2.2.2, page 66). Purity was assessed by flow cytometry, consistently 92-98% of cells were positive for CD3 and CD4 after gating on the viable lymphocyte population (Figure 3.1A). Cells were then cultured as unstimulated, anti-CD3-Ab or anti-CD3/28 treated cells for assessment of proliferation (Figure 3.1B, C) and cytokine production (Figure 3.1D-G). Cells were also harvested for extraction of total RNA to determine Notch pathway gene expression by RT-PCR (Figure 3.2).

$CD4^+$ T cell proliferation was measured at 24 hours and 48 hours. At 24 hours, little $[^3H]$ thymidine incorporation was observed by cells stimulated with anti-CD3 or anti-CD3/28 (Figure 3.1B). Stimulation of IL-2 production was measured at 24 hours also (Figure 3.1D). Secretion by unstimulated cells was minimal. Anti-CD3-Ab treatment induced low level IL-2 secretion increasing 3 fold when cells were activated with anti-CD3/28-Ab.

Proliferation measured at 48 hours revealed enhanced $[^3H]$ thymidine incorporation on stimulation with anti-CD3 alone compared to unstimulated cells, increasing further on stimulation with anti-CD3/28 (Figure 3.1C). Secretion of IFNγ, IL-4 and IL-10 was assessed after 48 hours of culture. Anti-CD3 stimulation induced IFNγ

(Figure 3.1E), IL-4 (Figure 3.1F) and IL-10 (Figure 3.1G) secretion by $CD4^+$ T cells, with only marginal enhancement in secretion on stimulation with anti-CD3/28 (Figure 3.1E, F, G respectively).

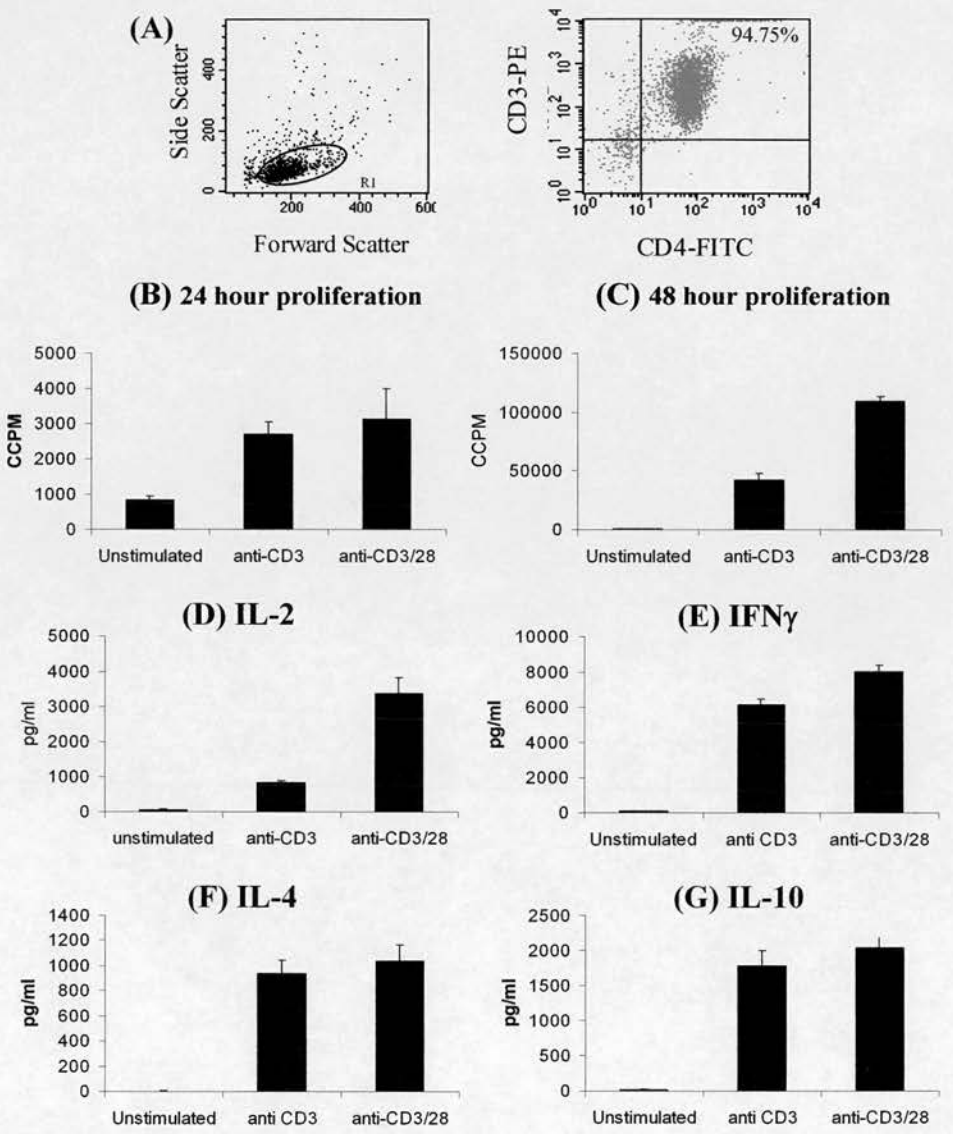


Figure 3.1: Purification and culture of $CD4^+$ T cells. Cells were purified using MACS $CD4^+$ T cells isolation kit with purities 95±3% cells positive for CD3 and CD4 (A). Proliferation on activation was measured at 24 and 48 hours by [^{3}H] thymidine incorporation (B, C). Cytokine secretion was measured by CBA analysis (IL-2, IFNγ, IL-4) or ELISA (IL-10)(D, E, F, G, F). Data shown is representative of four experiments.

Having established a purification protocol and demonstrated that the derived $CD4^+$ T cells were receptive to CD3 and CD3/CD28 ligation, total RNA was extracted. Transcript expression of *notch* receptors *1*, *2*, *3* and *4* were detected in cells rested or activated with anti-CD3 or anti-CD3/28 (Figure 3.2) over 48 hours. Expression of the ligands *jagged1*, *2* and *delta-like1* were also detected (Figure 3.2). The Notch target gene *hes1* was not observed to be expressed by resting cells but transcripts were detected in anti-CD3 and anti-CD3/28 T cells (Figure 3.2).

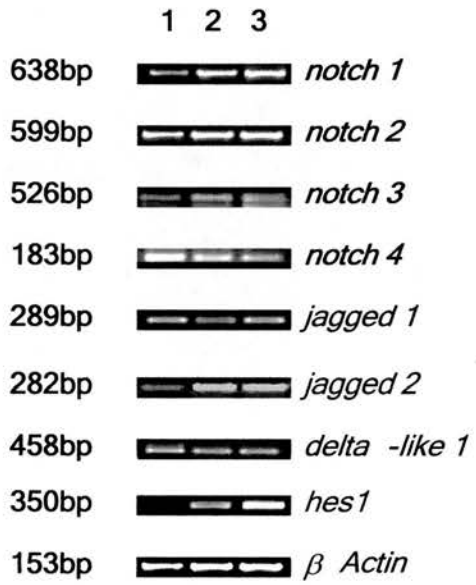


Figure 3.2: Expression of Notch pathway components by $CD4^+$ T cells. RT-PCR was used to demonstrate Notch pathway gene expression, with lanes 1, 2 and 3 representing unstimulated, anti-CD3 and anti-CD3/28 treated cells respectively. Details of primer sequences and annealing temperatures can be found in Materials and Methods (Chapter 2.2.7, page 73).

3.3 Activation of $CD4^+$ T cells induces differential transcript expression of Notch pathway components

RT-PCR revealed expression of Notch pathway components by unstimulated, anti-CD3 and anti-CD3/28 activated $CD4^+$ T cells. T cell activation activates the Ras and NFκB pathways, both developmentally linked to alteration of Notch signal transduction and expression of Notch receptors and ligands (95,120,121). RT-PCR data suggested that $CD4^+$ T cell activation might indeed affect the degree to which Notch pathway component genes are expressed in this cell type (seen as variations seen in band intensity, particularly *hes1*). However, this method is somewhat insensitive and consequently real-time RT-PCR was used instead. This method allowed representation of gene expression by anti-CD3 and anti-CD3/28 treated $CD4^+$ T cells relative to unstimulated cells and a clear quantification of increases or decreases in gene expression.

Cells were isolated and stimulated as before, and total RNA was reverse transcribed into cDNA for analysis by real-time RT-PCR. Data was calculated as a relative value, having been calibrated back to unstimulated cells (Chapter 2.2.8, page 75). A relative 2-fold change or more was considered biologically relevant (Chapter 2.2.8, page75). A list of primers and probes used can be found in the Materials and Methods Section (Chapter 2.2.8, page 75)

The Notch target gene *hes1* was used as a read-out of the canonical Notch signalling pathway. The influence Notch signalling normally has on *deltex* is unclear, but

expression was analysed to characterise this pathway in $CD4^+$ T cells. Transcription of *hes1* was enhanced significantly upon T cell activation in three out of three experiments. An average relative expression of 5.3±0.9 in anti-CD3-Ab treated groups (Figure 3.3A) and 4.4±1.5 in anti-CD3/28-Ab treated groups (Figure 3.3A) was observed relative to unstimulated cells. Unlike *hes1*, *deltex* transcript signals were observed to decrease upon activation, this decrease neared biological relevance when cells were treated with anti-CD3-Ab alone, dropping to 0.67±0.3 (Figure 3.3B) from a relative unstimulated value of 1. The reduction in signal only became significant on stimulation with both anti-CD3- and anti-CD28-Ab, falling to 0.36±0.08 (Figure 3.3B).

Analysis of Notch receptor gene expression did not reveal significant changes in either *notch1* or *notch2* expression (Figure 3.3C and D respectfully) after T cell activation with either anti-CD3- or anti-CD3/28-Ab. Relative expression remained similar to those observed for unstimulated cells in two out of three experiments. Increased signals for *notch1* and *notch2* upon T cell activation reached biological relevance in one individual experiment, but this was not reproducible. Expression of *notch3* and *notch4* decreased on activation with anti-CD3-Ab, dropping to 0.3±0.13, and 0.26±0.32 respectively (Figures 3.3E and F). Stimulation with anti-CD3/28-Ab saw a further reduction relative to unstimulated cells, falling to 0.15±0.07 and 0.19±0.2 for *notch3* and *notch4* respectfully (Figures 3.3E and F).

Transcript expression relative to unstimulated cells for the Notch ligands *jagged1, 2* and *delta-like1* were all observed to decrease significantly on either anti-CD3- or

anti-CD3/38-Ab activation. Expression of *jagged1* fell to 0.24±0.1 where cells were stimulated with anti-CD3-Ab and to 0.14±0.02 in groups stimulated with anti-CD3/28-Ab (Figure 3.3G). *jagged2* expression also decreased upon activation, with anti-CD3-Ab stimulation resulting in a relative *jagged2* expression value of 0.3±0.05 and 0.3±0.07 upon anti-CD3/28-Ab stimulation (Figure 3.3H). This downward trend was also observed for expression of *delta-like1*, with a substantial drop in relative expression values to 0.09±0.1 where cells were anti-CD3-Ab treated and to 0.13±0.12 on anti-CD3/28-Ab activation (Figure 3.3I).

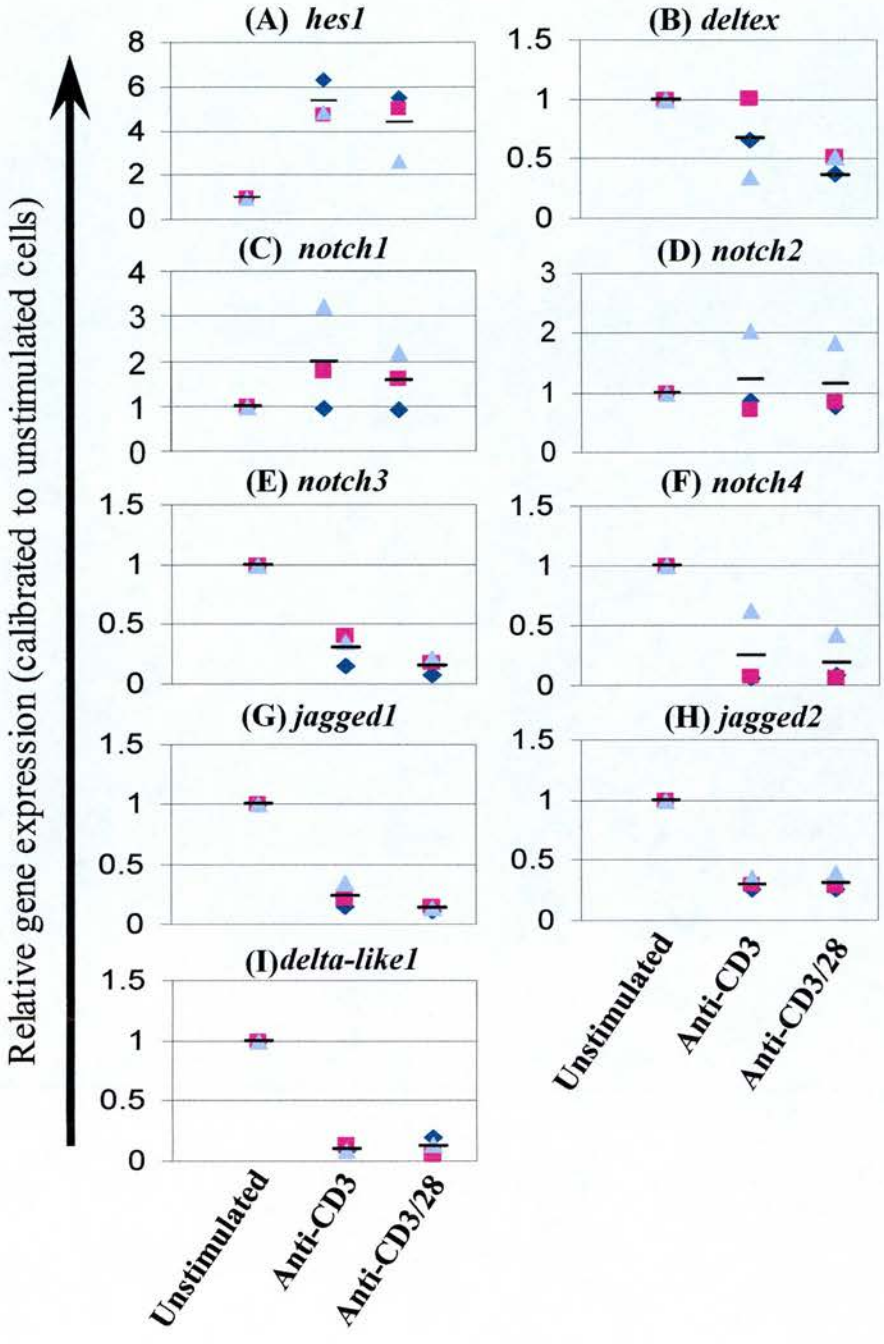


Figure 3.3: Changes in Notch pathway component expression after CD4$^+$ T cell activation. RNA from unstimulated, anti-CD3, or anti-CD3/28 treated CD4$^+$ T cells was harvested after 48 hours of *in vitro* culture, reverse transcribed and gene expression assessed by real-time RT-PCR. Data is presented relative to unstimulated cells. Three individual experiments are each represented as one colour of icon; the average value is depicted as a black bar.

3.4 Notch1 co-localises with CD4 upon T cell activation

On T cell activation, signal transduction molecules aggregate forming supramolecular activation clusters (SMACs). TCR induced SMAC formation aids T cell activation, stabilising and enhancing signal transduction (2). Recruitment of co-stimulatory molecules to the forming synapse further influences stabilisation and augmentation of TCR signal transduction and consequently contributes to the response made to antigenic stimulation. If Notch receptors are to influence T cell activation/effector function directly, these receptors might be expected to localise with TCR signalling apparatus. CD4 is recruited to the immunological synapse (125), stabilising TCR/MHC II interactions by binding directly to the MHC class II molecule. To assess the spatial distribution of Notch receptors upon T cell activation, resting and activated T cells were assessed for expression of CD4 and Notch1 by confocal microscopy. Co-localisation of these two molecules would imply Notch1 is also a component of the synapse, along with the TCR. The rat anti-mouse CD4 Ab L3T4 was used in conjunction with a goat anti-rat Alexa Fluor 594 (red). Notch staining was demonstrated using a FITC conjugated mouse anti-Notch1 antibody, designated A6, in conjunction with a rabbit anti-FITC Ab followed by a goat anti-rabbit Alexa Fluor 488 (green). Cells were counter stained with ToPro3 (blue), and examined using a Leica TCS MT confocal system.

Studies examining immune synapse formation have exhibited T cell polarisation within 30 minutes of T cell interaction with target APC. Others have also demonstrated similar time scales using soluble anti-CD3-Ab cross-linked with

fluorescent secondary antibodies. To determine an appropriate time-point to harvest cells for visualisation of T cell capping, unstimulated cells, cells activated for 30minutes, 16 hours and 24 hours were examined for polarisation of CD4 (red). Initial staining of resting $CD4^+$ T cells revealed a diffuse pattern throughout the cell membrane (Figure 3.4A). Polarisation of CD4 was not evident 30 minutes after activation with plate-bound anti-CD3- and soluble anti-CD28-Ab (Figure 3..4B). Capped CD4 staining was observed at 16 hours post activation (Figure 3.4C). Activated cells stained at 24 hours were visually larger than unstimulated cells and activated cells at 30 minutes, however, CD4 polarisation was not evident (Figure 3.4D), perhaps consistent with disruption of synapses as cells prepare for division.

Cells were also examined for Notch1 expression at these time points (Figure 3.5). As with CD4 staining, Notch1 was found to be dispersed throughout the membrane in resting cells (Figure 3.5A). This staining profile did not alter after cells had been activated for 30 minutes with anti-CD3/28-Ab (Figure 3.5B). By 16 hours post activation, cells exhibiting polarised Notch1 staining were observed (Figure 3.5C). Notch1 was not seen to cap in cells stained 24 hours after activation (Figure 3.5D).

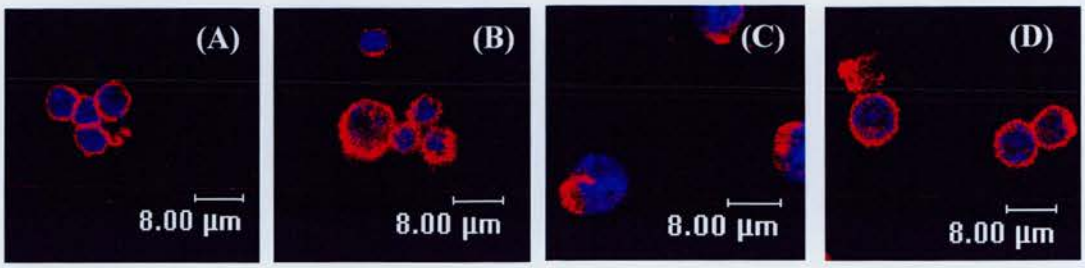


Figure 3.4: CD4 capping. Unstimulated cells (A) were stained for CD4 (red) and after 30 minutes (B), 16 hours (C) and 24 hours (D) anti-CD3/28 stimulation. Cells were counterstained with ToPro3 (blue).

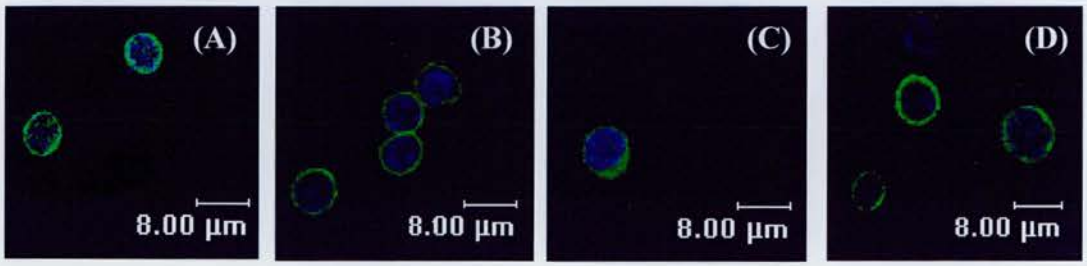


Figure 3.5: Notch1 capping. Unstimulated cells (A) were stained for Notch1 (green) and after 30 minutes (B), 16 hours (C) and 24 hours (D) anti-CD3/28 stimulation. Cells were counterstained with ToPro3 (blue).

With both CD4 and Notch1 being seen to cap by 16 hours, this time-point was chosen to examine co-localisation of these molecules. Unstimulated and anti-CD3/28-Ab activated cells were cultured for 16 hours, harvested and stained for Notch1 and CD4. Co-localised emissions of 488 and 594 appear as yellow, indicating areas where CD4 and Notch1 are in close proximity. No evidence of co-localisation was found on overlay of unstimulated cell stains (Figure 3.6A, B and C), as revealed by lack of yellow colouring (Figure 3.6C) and confirmed on analysis of fluorescent pixel intensity in relation to cell surface position (Figure 3.6E).

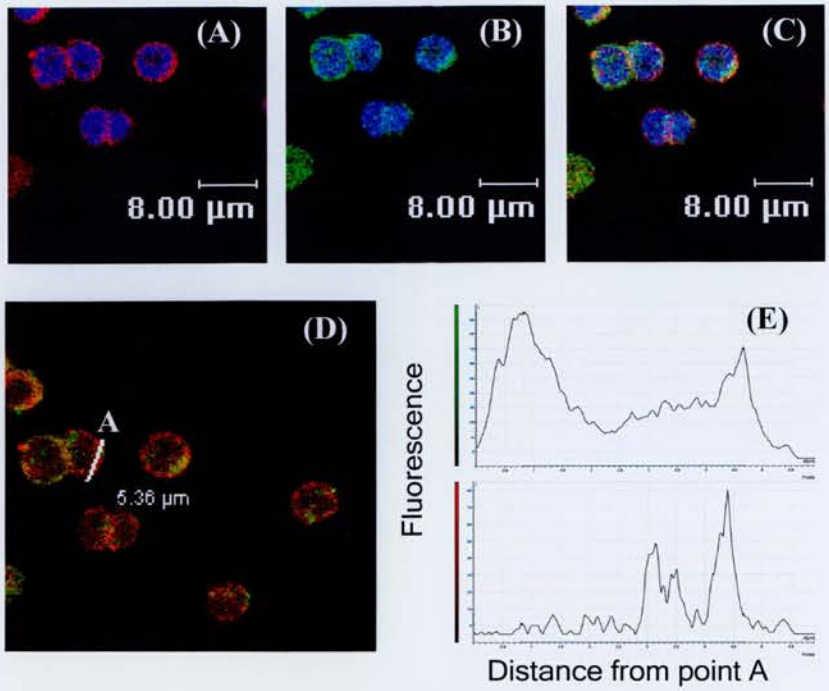


Figure 3.6: Notch1 and CD4 do not co-localise on resting T cells. Figures show surface staining of CD4 (red) (A), Notch1 (green) (B) and an overlay (C) respectively by unstimulated $CD4^+$ T cells cultured for 16 hours. Cells are counter stained with ToPro3 (blue). Co-localization was assessed by analysing pixel fluorescent intensity relative to distance from point A, marked in figure (D). Figure (E) depicts this data in graphical form. Data shown is representative of four experiments.

Analysis of anti-CD3/28-Ab activated T cells revealed polarisation of CD4 and Notch1 as previously described (Figures 3.4 and 3.5 respectively). Overlay of both stains revealed localisation to the same area, seen as yellow (Figure 3.7C). Analysis of pixel intensity for both red and green fluorescence in relation to their position on the cell surface revealed correlation in the position of the major peaks of fluorescence, confirming co-localisation of CD4 and Notch1 (Figures 3.7D and E) in the T cell cap. 92±4% of the 153 capped cells examined were found to exhibit co-localisation of CD4 and Notch1. Isotype controls and single stains did not demonstrate cross-reactivity of the two staining protocols (Figure 3.8, Table 3.1).

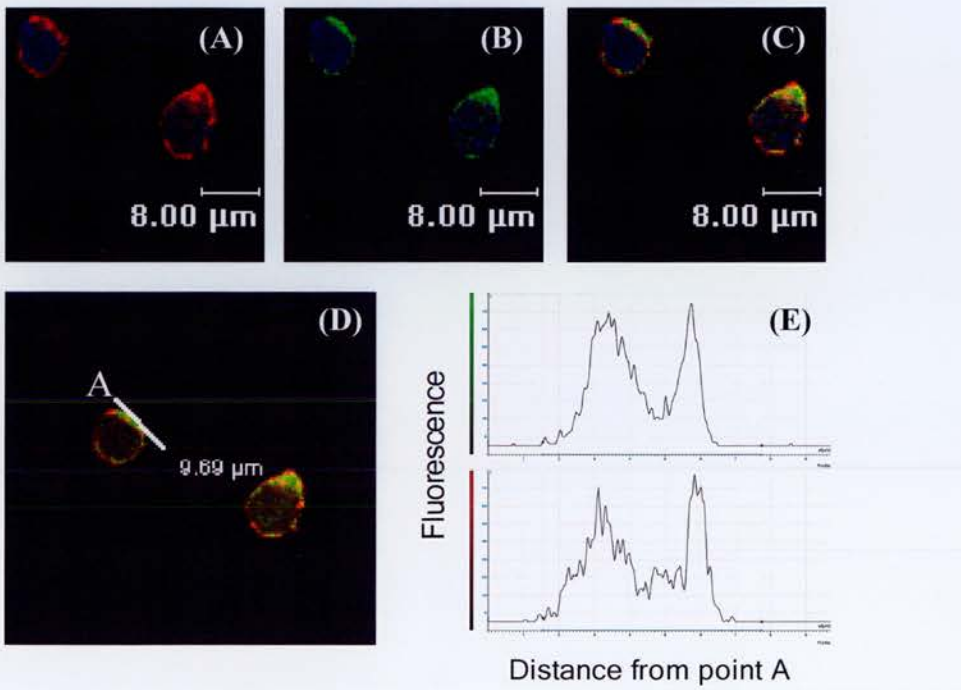


Figure 3.7: Co-localisation of Notch1 and CD4 upon T cell activation. Figures show surface staining of CD4 (red) (A), Notch1 (green) (B) and an overlay (co-localization, yellow) (C) respectively by anti-CD3/28-Ab activated $CD4^+$ T cells. Cells are counter stained with ToPro3 (blue). Co-localization was confirmed by assessing pixel fluorescent intensity against distance from point A, marked in figure (D). Figure (E) depicts this data in graphical form. Data shown is representative of five experiments.

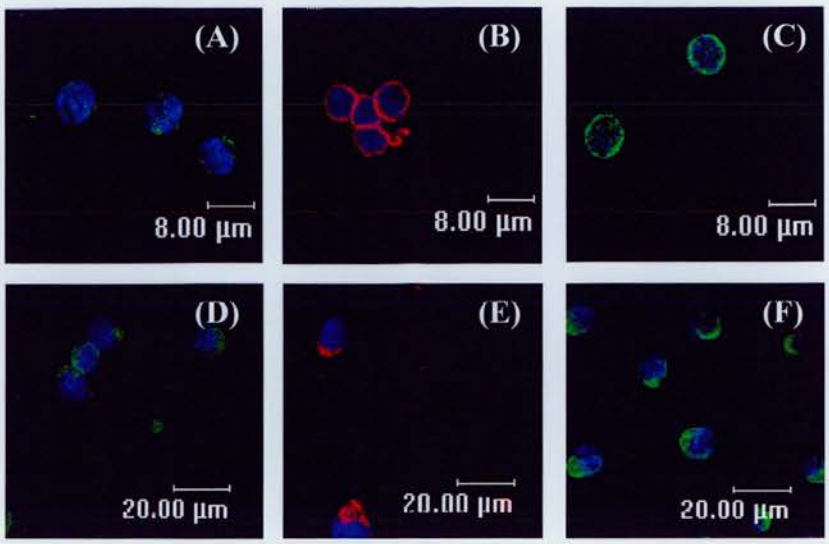


Figure 3.8: Control staining: Panels display resting cells (A, B, C) and anti-CD3/28 activated cells (D, E, F). Negative controls (A, D); CD4 single stain (red) testing cross-reactivity with Notch secondary and tertiary antibodies (B, E); Notch1 single stain (green) testing cross-reactivity with CD4 staining secondary (C, F) are depicted. For details of antibodies used in each panel, see table 3.1.

Table 3.1: Key for Figure 3.8: Control staining.

Panel	Treatment	Isotype	Primary	Anti-rat Alexa Fluor 594	Rabbit anti-FITC	Anti-rabbit Alex Fluor 488
(A)	resting	rat IgG2bκ mIgG2bκ–FITC	no	yes	yes	yes
(B)	resting	no	CD4 (L3T4)	yes	yes	yes
(C)	resting	no	Notch1 (A6)	yes	yes	yes
(D)	anti-CD3/28	rat IgG2bκ mIgG2bκ–FITC	no	yes	yes	yes
(E)	anti-CD3/28	no	CD4 (L3T4)	yes	yes	yes
(F)	anti-CD3/28	no	Notch1 (A6)	yes	yes	yes

3.5 Discussion

The results presented here establish transcript expression of Notch receptors and ligands by mature murine $CD4^+$ T cells. These genes are differentially expressed upon T cell activation. Notch1 surface staining further confirmed receptor expression but also revealed co-localisation with CD4 upon T cell stimulation with anti-CD3/28-Ab.

Expression of Notch receptors and the demonstration that Notch signal transduction, as measured by *hes1* transcription, occurs in $CD4^+$ T cells supports a model in which Notch ligands expressed by APCs engage Notch receptors on T cells and modulate their activation, effector function and/or differentiation (1,126,127, 105). Stimulation of $CD4^+$ T cells with anti-CD3- or anti-CD3/28-Ab reduced transcription of *notch3*, *notch4*, *jagged1*, *jagged2* and *delta-like1*. This was an unusual finding. It might have been expected that ligand expression would have increased upon T cells activation. TCR/CD28 signalling activates both NFκB and Ras pathways, known to increase transcription of *jagged1* and *delta-like1* respectively (120, 95, 121). Increased levels of *hes1* indicate enhanced Notch signal transduction. Classically cells receiving a Notch signal down-regulate expression of Notch ligands, as observed in lateral inhibition (128, 129) and this may explain the reduced ligand expression observed here. Notch receptors are believed to differ in their affinity for ligands and in their ability to induce transcription of *hes1* (130). T cell activation may promote *hes1* transcription by altering which receptors/ligands they express. To help elucidate possible mechanisms as to how these genes are differentially regulated, a time-course

encompassing early time-points would be advantageous. This would allow comparison to the timing of known TCR signalling events and possibly highlight particular pathways in regulation of Notch related genes.

RT-PCR and real-time RT-PCR were effective in establishing that Notch pathway component genes are expressed by $CD4^+$ T cells. Ideally, this study would have expanded on the RNA derived data by examining protein expression, perhaps by Western blot. Although some antibody reagents raised against components of the Notch pathway are available, attempts to demonstrate protein expression by Western blot or flow cytometry have been unsuccessful. This is not to say that the proteins of interest are not present, rather that attempts to establish effective protocols to detect them have been unsuccessful. Use of the anti-Notch1 monoclonal antibody with various Western blot techniques proved fruitless, but did however demonstrate staining by confocal microscopy. This detection of Notch1 upon $CD4^+$ T cells corroborates the RT-PCR data.

Activation of mature T lymphocytes induces aggregation of lipid rafts, containing molecules known to influence TCR signalling, and formation of the immunological synapse (2, 131). Activation induced recruitment of Notch1 to the synapse could potentially modulate TCR signal transduction. CD4 was used as a marker for polarisation of T cells upon activation, this molecule is known to interact with the TCR, stabilising its initial interaction with MHC class II molecules (132) and enhancing signal transduction by recruitment of Lck (133). Confocal microscopy revealed Notch1 did not to co-localise with CD4 on unstimulated T cells, implying

that these molecules are not in the same lipid rafts. Activation with anti-CD3/28-Ab induced polarisation of Notch1 and CD4, seen as co-localisation in the T cell cap. Although this does not show direct interaction between the Notch receptor and TCR, the implication is there. Notch and TCR association would support the existing theory that Notch attenuates TCR signal transduction in thymocytes, where over-expression of NICD1 inhibited CD5 and CD69 up-regulation (99). Notch is also capable of negatively regulating Ras/JNK (120, 134, 135), AP-1 (136, 137) and NFκB activity (138, 139), all of which are utilised in the activation of T cells. In this context does Notch serve to resolve T cell activation, as with CTLA-4 or PD-1 (9)? Direct induction of Notch signalling may represent a means of aborting T cell activation.

Immunological synapses are the formation of a junction between T cell and APC. The use of anti-CD3/28-Ab to induce T cell polarisation in the absence of APCs could be viewed as being particularly artificial. However, findings from such systems appear to correlate with those using APCs. By activating T cells in this fashion, capping of Notch1 has been driven by the T cell itself rather than being recruited by Notch ligand bearing APCs, possibly representing a requirement for Notch signalling upon T cell activation. Attempts to visualise T cell/APC interactions have been fraught with difficulty, particularly in preserving the contacts made between cells. This could be circumvented by staining *in vitro* and visualisation in real-time. The development of a directly conjugated anti-Notch1 antibody for use with confocal would be required. Closer examination of the time-scales associated with Notch1

recruitment to the immunological synapse would facilitate an understanding of its role in T cell activation.

3.6 Future work

To further enhance this study the following areas should be analysed. A detailed time-course examining differential expression of Notch related genes following activation would allow a more direct comparison to the now published literature regarding Notch1 and *hes1* expression by $CD4^+$ T cells. Time points of closer to 30 minutes or 1 hour following stimulation may indicate possible links to TCR signalling.

Protein expression information would be of considerable value and would compliment gene expression data. As more reagents are becoming available, this will facilitate further analysis of Notch pathway components by $CD4^+$ T cells. Attempts to detect surface expression of Delta-like1 have been made, but with limited success (Chapter 6.3, page 174). Use of Notch tetramers may prove useful in determining overall expression of ligand, but may prove difficult in tailoring to detect specific ligands.

Of particular interest was the finding that Notch1 and CD4 co-localise on activated T cells. However, the techniques employed to examine polarisation of T cells and particularly capturing images of APC/T cell interactions are very much in their infancy within the group. The acquisition of new computer software will facilitate a

detailed analysis of co-localisation on cells. FRET would be the method of choice if this aspect of the work is to be pursued. Additionally, generation of images showing APC/T cell interactions would allow for a more physiological interpretation of Notch and CD4 co-localisation. Some "real-time" images were generated in an attempt to visualise APC/T cell interactions, however this was very much a preliminary experiment and was limited due to technical resources.

4 Inhibition of Notch signalling

4.1 Introduction/background

Interactions between Notch receptors and their ligands results in regulated intramembrane proteolysis (RIP) (140). RIP is mediated by sequential processing by a disintegrin metalloprotease followed by presenilin/γ-secretase, resulting in shedding of extracellular domains and generation of soluble Notch intracellular domain (NICD). NICD translocates to the nucleus where interaction with transcriptional co-factors results in target gene expression (128). RIP also regulates the cleavage of the amyloid precursor protein (APP) in a similar way to that directing Notch signalling (64). APP processing yields amyloid β protein (Aβ) important in mediating the pathogenesis of Alzheimer's disease (141, 142). Presenilin/γ-secretase activity mediates the final proteolytic event generating Aβ fragments and as such this activity has been targeted as having therapeutic potential (142, 64). Numerous aspartyl protease transition state analogue inhibitors of γ-secretase have been developed that bind directly to presenilin subunits inhibiting γ-secretase complex function (143). In addition to inhibiting APP processing, Notch receptor cleavage is also blocked (64). While this has raised questions as to the validity of using γ-secretase inhibitors in Alzheimer's disease, it has provided an additional tool for studying Notch signalling.

Inhibition of Notch receptor signalling by pharmacological inhibitors of γ-secretase activity was demonstrated *in vitro* using a luciferase reporter activated by a ligand-independent truncated Notch receptor harbouring a GVP (Gal4 DNA-binding/VP16 transactivation) moiety (144). In the later study, use of γ-secretase inhibitors (specifically including that used in this thesis) decreased luciferase expression, and therefore γ–secretase activity, in a dose dependent manner. Further to this study, zebrafish embryo incubation with the potent γ-secretase inhibitor DAPT demonstrated phenotypes indistinguishable from Notch pathway mutants (145).

In two independent studies, γ-secretase inhibitors have been employed to interrupt Notch signalling in thymocytes, demonstrating similar effects on thymocyte development to those observed in conditional Notch knockout mice. These studies not only confirmed the use of γ-secretase inhibitors as a method of Notch inhibition in lymphoid cells but that strength of Notch signalling is also a relevant factor in directing thymocyte development.

The demonstration of co-localisation of Notch with CD4 on activated T cells (Chapter 3.4, page 93) and numerous documented interactions between Notch and components also utilised by TCR signalling implicate Notch as a potential modulator of T cell function. TCR induced activation of mature $CD4^+$ T cells is associated with proliferation and development of helper/effector function, predominantly mediated by cytokine secretion. TCR activation of NFAT/AP-1, NFκB and Ras activity induces transcription of IL-2, promoting clonal expansion, and is related to the

production of effector cytokines (e.g. IFNγ and IL-4). NFAT/AP-1 activity was shown to be attenuated by over-expression of Notch (99), while various developmental studies have implicated Notch as influencing NFκB and Ras activity (88, 100, 136, 137, 146, 147). Additionally, T-ALL studies demonstrate correlations in constitutive Notch activity (either Notch1 or Notch3) with increased proliferation and NFκB activity (88). If Notch does influence TCR signalling, it might be expected that inhibition of Notch signalling would affect T cell proliferation and/or cytokine secretion. The difluoroketone aspartyl protease transition state analogue MW167 was used to inhibit γ-secretase activity (63, 69, 142, 66), preventing endogenous cleavage of Notch receptors. Any alteration in $CD4^+$ T cell response to TCR stimulation in the presence of MW167 would formally link Notch to T effector function.

Chapter aims:

- Determine optimal dose of γ-secretase inhibitor (MW167) to prevent endogenous Notch signalling in unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells by *hes1* transcription
- Assess cell viability in the presence of MW167 to address any toxicity issues.
- Determine influence of Notch on $CD4^+$ T cell proliferative responses when unstimulated, anti-CD3- and anti-CD3/38-Ab treated.
- Characterise cytokine secretion profiles of unstimulated and activated $CD4^+$ T cells in the absence of Notch.
- Address possible transcriptional regulation of T effector function by Notch.

4.2 γ-secretase inhibitor reduces Notch signalling as measured by *hes1* expression

The γ-secretase inhibitor MW167 was chosen for this study as unlike some inhibitors of γ-secretase activity it is not a proteasome inhibitor. Proteasome inhibition has been used to alter Notch signalling. However, as this may have non-specific effects on T cell biology such inhibitors were avoided. At low doses MW167 specifically inhibits generation of presenilin (PS) N-terminal (NTF) and C-terminal fragments (CTF) from full-length presenilin (PS FL), and at higher doses also inhibits activity of the γ-secretase complex (69). MW167 also functionally blocks generation of NICD, confirming its use in blockade of Notch signal transduction (144). The concentrations used in this study are based on the published IC50 for MW167 and concentrations used in developmental systems that maximally inhibit NICD generation (144, 69, 66).

Real-time RT-PCR was used to determine $CD4^+$ T cell expression of the canonical Notch target gene *hes1* in the presence of increasing concentrations of MW167. Expression was assessed in unstimulated, anti-CD3- and anti-CD3/28-Ab treated cells, in the absence or presence of the γ-secretase inhibitor MW167 at both 24 and 48 hours. 2 fold changes in relative gene expression were considered biologically relevant.

Increased *hes1* transcripts were detected in unstimulated $CD4^+$ T cells when treated with 5μM MW167 for 24 hours compared to untreated cells, with a mean relative

expression of 4.7±2.1 (Figure 4.1A). Little change in *hes1* expression was also observed in unstimulated 5μM 48 hour cultures, relative expression 1.4±0.5 (Figure 4.1B). Increasing MW167 concentrations to 10μM and 20μM did not induce a biologically relevant decrease in *hes1* transcription in unstimulated 24 hour cultures (Figure 4.1A). Equivalent 48 hour cultures revealed decreased *hes1* transcript detection (Figure 4.1B). Reduced *hes1* expression in unstimulated 10μM MW167 48 hour cultures neared biological relevance with a mean relative expression 0.39±0.2 (Figure 4.1B). Increasing MW167 concentration to 20μM resulted in non-detection of *hes1* transcripts in two of 4 samples, and those detectable returning relative expression values of less than 0.04 (Figure 4.1B).

Stimulation of $CD4^+$ T cells with anti-CD3-Ab in the presence of MW167 revealed similar trends in regulation of *hes1* transcription to those observed in unstimulated cultures (Figure 4.1C, D). In 24 hour anti-CD3-Ab cultures, incubation with 5μM MW167 increased mean *hes1* expression relative to stimulated cells without MW167 (Figure 4.1C). Relative *hes1* expression appeared unaffected at 10μM (0.8±0.3) but fell to a mean of 0.3±0.08 when anti-CD3-Ab treated cells were incubated with 20μM MW167 for 24 hours (Figure 4.1C).

No biologically relevant alteration in *hes1* transcription was detected at either 24 or 48 hours when anti-CD3/28-Ab stimulated cells were cultured with 5μM MW167. At 10μM MW167, a downward trend in relative *hes1* expression was observed at both 24 and 48 hours, mean relative expression values of 0.67±0.3 and 0.3±0.1

respectively (Figure 4.1E, F). Incubation of anti-CD3/28-Ab stimulated cells with 20μM MW167 for 24 and 48 hours prevented biologically relevant amounts of *hes1* transcription, reducing mean relative expression to 0.25±0.1 and 0.22±0.09 respectively.

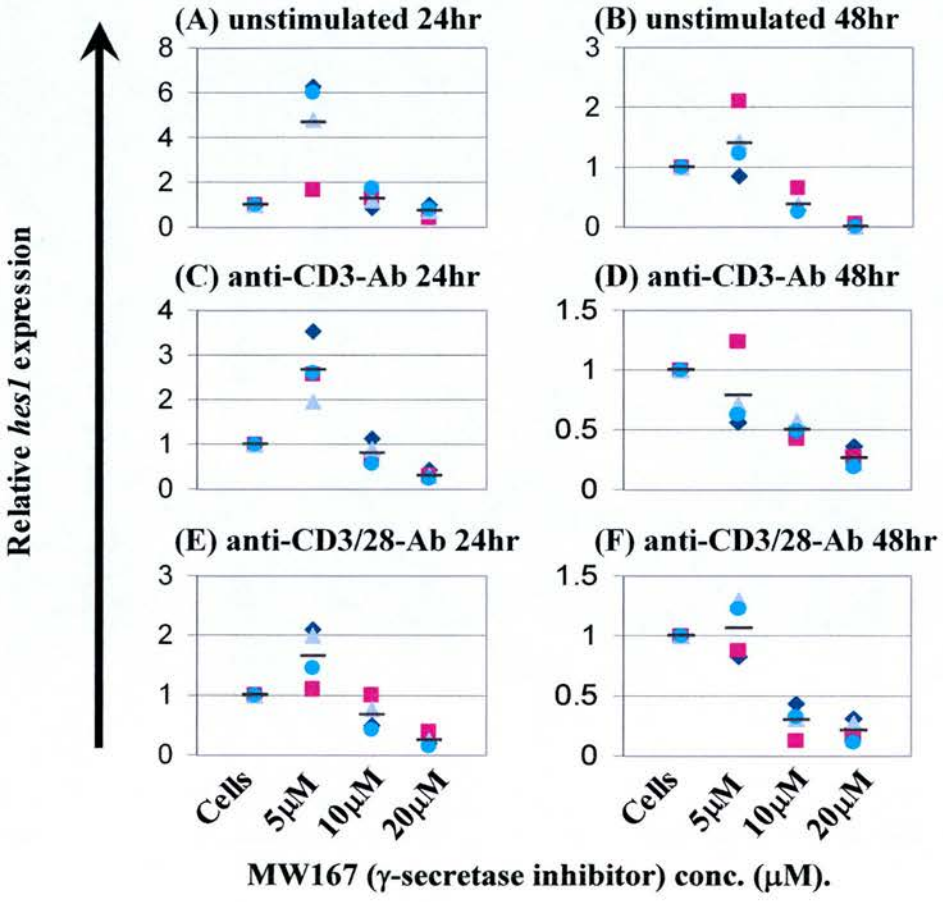


Figure 4.1: Reduced *hes1* expression by CD4$^+$ T cells on culture with γ-secretase inhibitor. Unstimulated, anti-CD3- or anti-CD3/28-Ab treated CD4$^+$ T cells were cultured for 24 or 48 hours with DMSO carrier control (equivalent to the amount present with the maximal inhibitor dose) or with increasing quantities of γ-secretase inhibitor (MW167). Real-time RT-PCR was then used to determine expression of *hes1* relative to cells control. Four individual experiments are each represented as one colour of icon; the average value is depicted as a black bar.

4.3 γ-secretase inhibitor does not affect $CD4^+$ T cell viability

Notch signalling has been related to enhanced survival of thymocytes undergoing negative selection by providing increased resistance to glucocorticoid induced apoptosis (119). This effect has not been attributed to one particular Notch receptor, with Notch1 deficiency not affecting viability of developing thymocytes, implicating redundancy between the Notch family receptors (148). Given that γ-secretase inhibitors are not believed to be Notch receptor selective in inhibition of signalling, $CD4^+$ T cell viability in the presence of MW167 was assessed.

Purified $CD4^+$ T cells were cultured in the absence or presence of 20μM MW167, the dose at which *hes1* expression was retarded most. Cells were unstimulated, anti-CD3- or anti-CD3/28-Ab treated for 48 and 72 hours. Cells were then harvested and stained with annexin V and 7AAD. Flow cytometric analysis did not reveal significant differences in cell viability between cells cultured with DMSO carrier or 20μM MW167 after 48 or 72 hours (Figures 4.2 and 4.3 respectively).

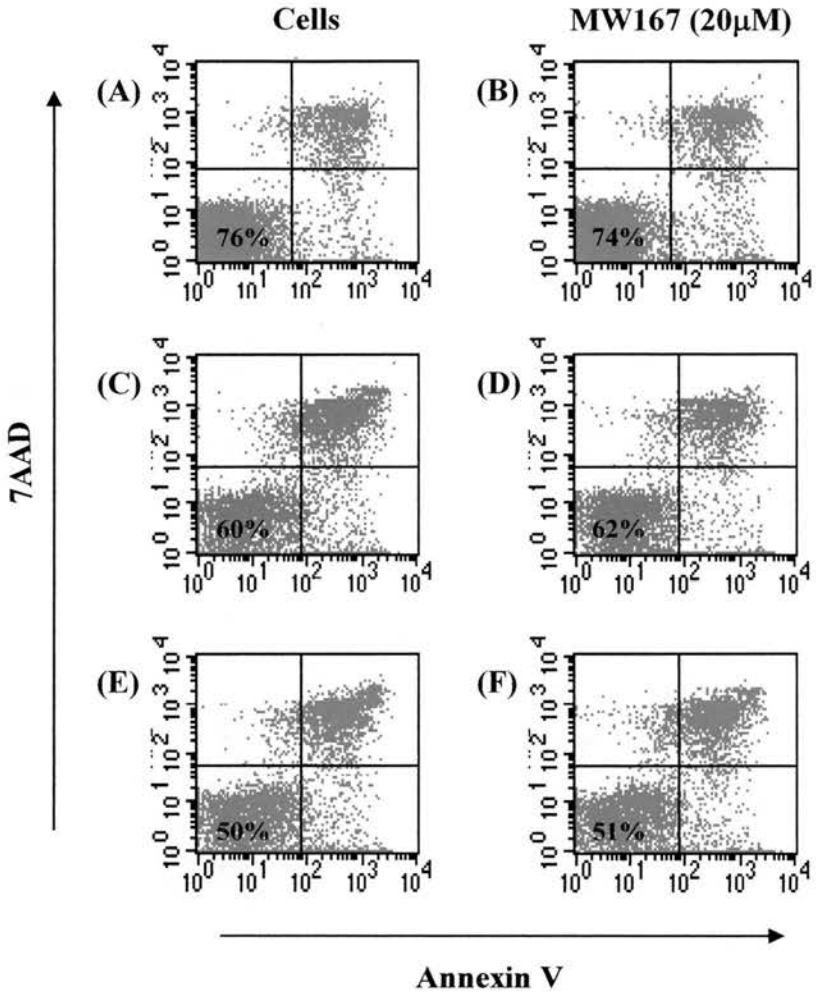


Figure 4.2: Cell viability is unaffected by γ-secretase inhibitor (48 hours). Cell viability for unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells was assessed by Annexin V/7AAD staining, after 48 hours. Figures A, C and E: cells cultured with DMSO. Figures B, D and F: cells incubated with 20μM MW167. Data is representative of three individual experiments.

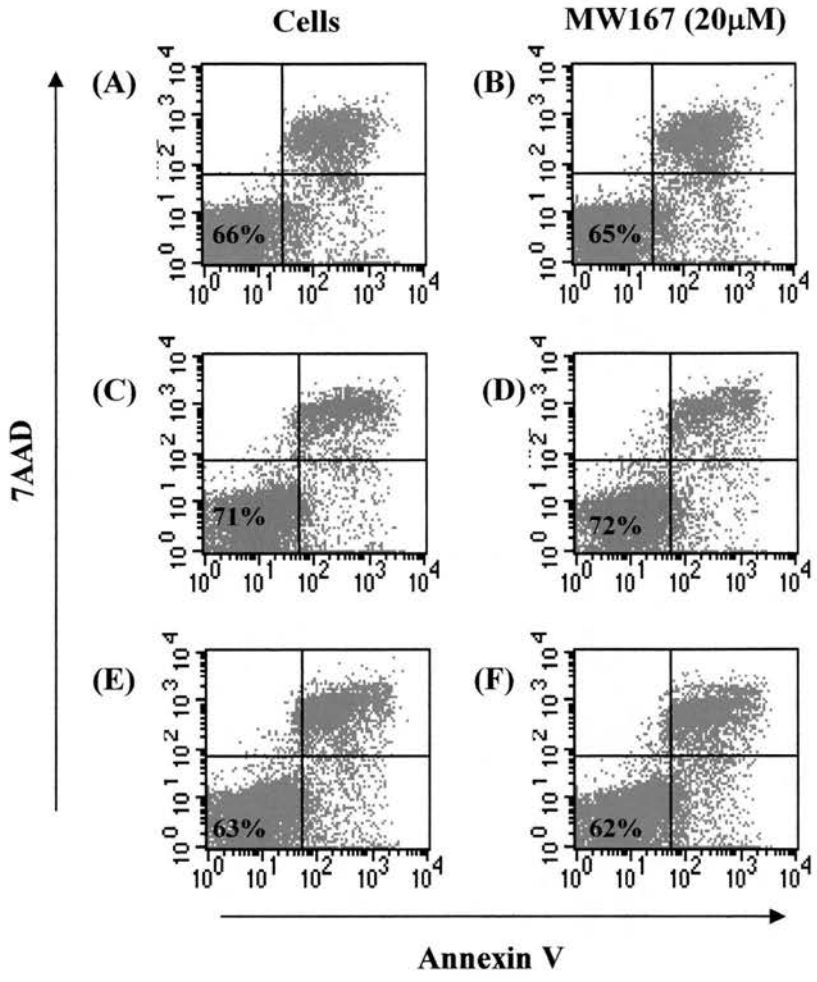


Figure 4.3: Cell viability is unaffected by γ-secretase inhibitor (72 hours). Cell viability for unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells was assessed by Annexin V/7AAD staining, after 72 hours. Figures A, C and E: cells cultured with DMSO. Figures B, D and F: cells incubated with 20μM MW167. Data is representative of three independent experiments.

4.4 $CD4^+$ T cell proliferation is unaffected by inhibition of Notch signalling

The association of neoplastic T cell proliferation and enhanced Notch activity is well documented. Based in this, it was decided to examine normal $CD4^+$ T cell proliferative responses in the absence of Notch signalling. Purified BALB/c $CD4^+$ T cells were cultured in the presence of increasing doses of the γ-secretase inhibitor MW167. Cells were unstimulated, anti-CD3- or anti-CD3/28-Ab treated and cultured for 48 or 72 hours. Proliferation was measured using [^{3}H] thymidine incorporation.

At the concentrations tested, MW167 was not observed to affect proliferation of unstimulated, anti-CD3- or anti-CD3/28-Ab treated $CD4^+$ T cells (Figure 4.4). This was observed after both 48 and 72 hours of culture.

In conjunction, secretion of the T cell mitogen IL-2 was also assessed. The concentration of IL-2 in supernatants from unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells in the absence or presence of 20μM MW167 were assayed by cytometric bead analysis at 24 hours (Figure 4.5). IL-2 was undetectable in unstimulated cultures. Low level IL-2 was detected in anti-CD3-Ab stimulated cultures, but was unaffected by MW167. Stimulation with anti-CD28- in addition to anti-CD3-Ab significantly enhanced IL-2 secretion ($p=0.0002$) but again was unaffected by MW167.

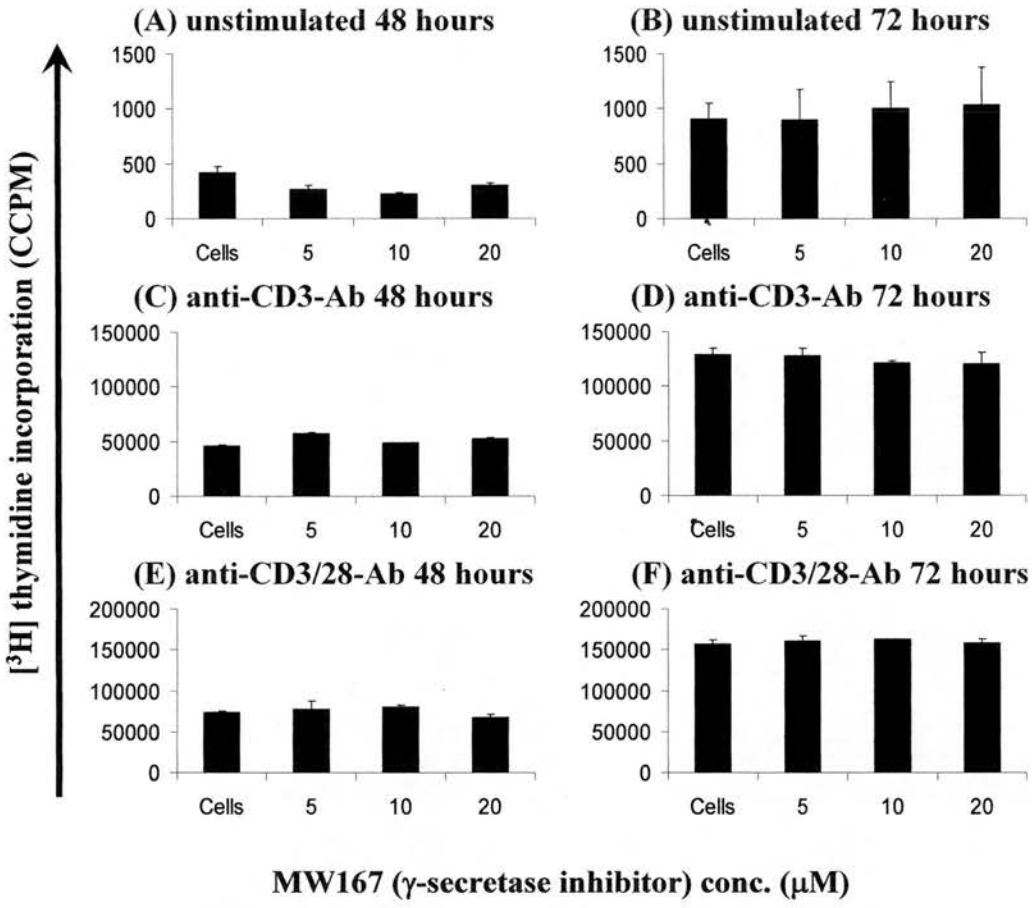
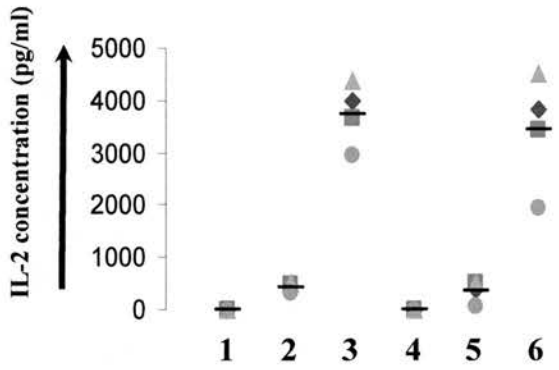


Figure 4.4: CD4$^+$ T cell proliferation in the absence of Notch signalling. Proliferation by unstimulated (A, B), anti-CD3- (C, D) and anti-CD3/28-Ab (E, F) treated CD4$^+$ T cells was assessed by [^{3}H] thymidine incorporation at both 48 (A, C, E) and 72 hours (B, D, F).



1: unstimulated cells, DMSO
2: anti-CD3 cells, DMSO
3: anti-CD3/28 cells, DMSO
4: unstimulated cells, MW167 (20μM)
5: anti-CD3 cells, MW167 (20μM)
6: anti-CD3/28 cells, MW167 (20μM)

Figure 4.5: IL-2 secretion in the absence of Notch signalling by $CD4^+$ T cells. Cytometric bead analysis was used to assess release of IL-2 by unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells in the absence or presence of 20μM MW167. Four individual experiments are each represented as one colour of icon; the mean value is depicted as a black bar.

4.5 Notch signalling is required for cytokine secretion in the absence of CD28 co-stimulation

To further investigate a role for Notch in $CD4^+$ T cell effector function, cytokine secretion was analysed in the absence or presence of the γ–secretase inhibitor. Unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells were cultured for 48 or 72 hours with either carrier DMSO or with 20μM MW167. TNFα, IFNγ, IL-4 and IL-5 secretion were assessed by cytometric bead analysis.

Secretion of TNFα, IFNγ, IL-4 or IL-5 by unstimulated cells was not detectable at either 48 hours or 72 hours in the absence or presence of MW167 (Figure 4.6).

Secretion of TNFα and IFNγ was up-regulated upon 48 hour activation with immobilised anti-CD3-Ab alone (Figure 4.6A, B). Addition of 20μM MW167 inhibited secretion of these Th1 cytokines, reducing detectable TNFα levels by 53.7±5.5% ($p=0.0007$) and IFNγ by 64.75±5.9% ($p=0.01$) (Figure 4.6A, B). However, anti-CD3/28-Ab activated cells secreted comparable TNFα and IFNγ when cultured in the presence or absence of MW167 (Figure 4.6A, B). Secretion of the Th2 cytokines IL-4 and IL-5 following anti-CD3-Ab stimulation in the presence of Notch inhibitor was also repressed, with reductions of 71±9.8% ($p=0.005$) and 73.4±9.3 ($p=0.007$) respectively (Figure 4.6C, D). TCR ligation together with anti-CD28 co-stimulation in the absence of Notch signalling restored IL-4 and IL-5 levels (Figure 4.6C, D).

TNFα and IFNγ were similarly found in supernatants collected after 72 hours of culture with anti-CD3- and anti-CD3/28-Ab stimulation. Incubation in the presence of MW167 reduced release of TNFα by 72.9±6% (p=0.026) and IFNγ by 57.2±11% (p=0.003) when cells were stimulated with anti-CD3-Ab alone (Figure 4.6E, F). When cells were stimulated with anti-CD3/28-Ab, secretion of these Th1 cytokines appeared equivalent between control and MW167 groups (Figure 4.6E, F). As with 48 hour cultures, a trend for reduced IL-4 and IL-5 secretion upon anti-CD3-Ab stimulation in the presence of MW167 was apparent in 72 hour cultures (Figure 4.6G, H). Reductions in IL-4 and IL-5 secretion in anti-CD3-Ab plus MW167 groups were found to be significant (p=0.02 and p=0.04 respectively). Stimulation with anti-CD3/28-Ab with 20μM γ-secretase inhibitor did not significantly alter secretion of these Th2 cytokines (Figure 4.6G, H).

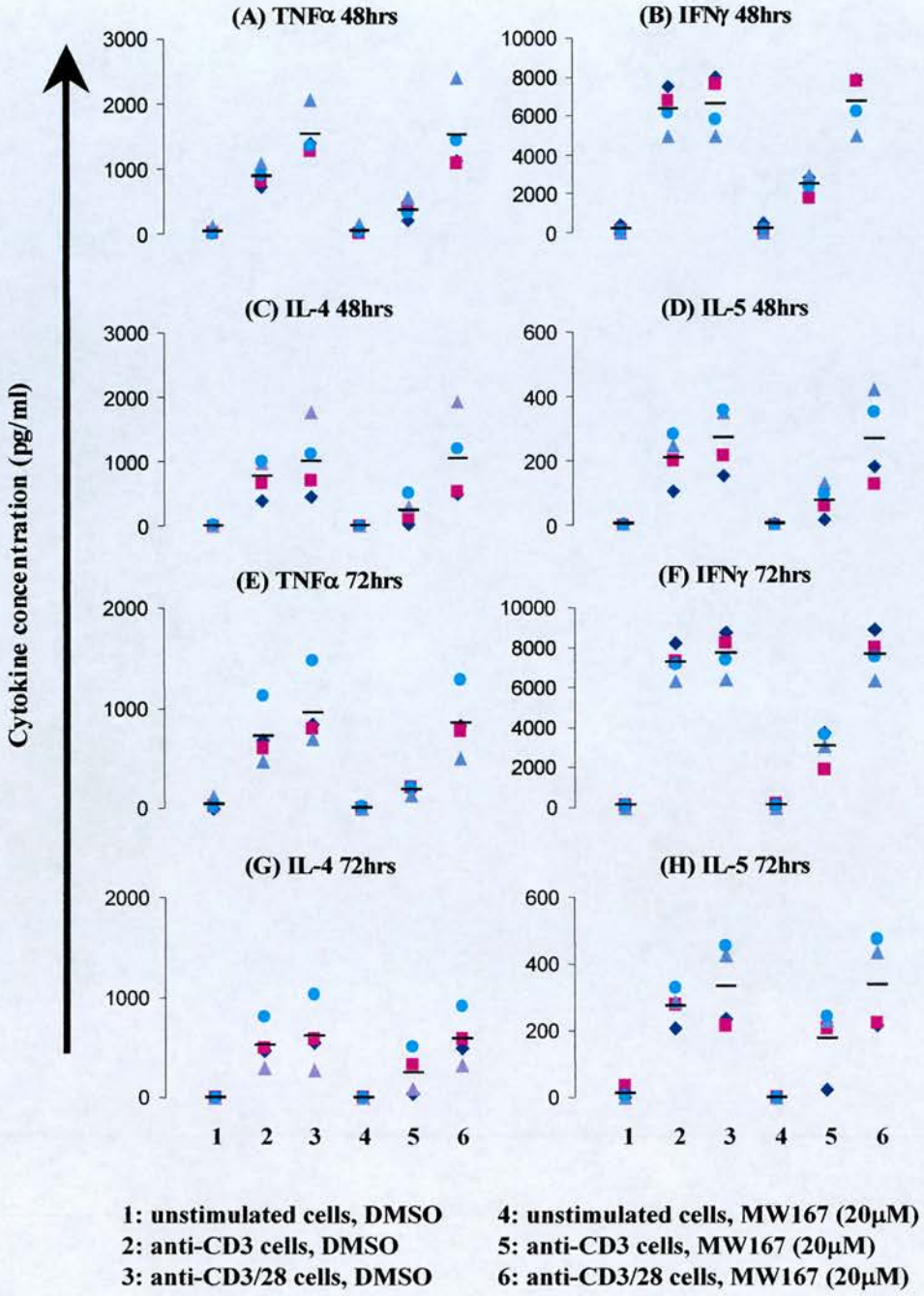


Figure 4.6: Cytokine secretion by anti-CD3- but not anti-CD3/28-Ab stimulated CD4$^+$ T cells is impaired in the absence of Notch signalling. Cytometric bead analysis was used to assess cytokine secretion by unstimulated, anti-CD3- or anti-CD3/28-Ab activated CD4$^+$ T cells cultured for 48 hours (A-D) and 72 hours (E-H) in the absence or presence of MW167 (γ-secretase inhibitor). Supernatants were assayed for TNFα (A, E), IFNγ (B, F), IL-4 (C, G), IL-5 (D, H). Four individual experiments are each represented as one colour of icon; the mean value is depicted as a black bar.

4.6 Notch signalling is required for IL-10 secretion even in the presence of CD28 co-stimulation

After examination of cytokine secretion relating to potential Th1 or Th2 effector function, the release of the immunoregulatory/Th2 cytokine IL-10 was assessed. This cytokine was of particular interest given the reported ability of APCs over-expressing Notch ligands to induce a regulatory T cell phenotype. IL-10 secretion by unstimulated cells, anti-CD3- and anti-CD3/28 treated $CD4^+$ T cells was determined by ELISA in the absence or presence of 20μM MW167.

IL-10 secretion by anti-CD3-Ab activated cells after 48 hour culture was impaired in the presence of MW167 (Figure 4.7A) with a mean reduction of 77±8.3% in IL-10 levels compared to anti-CD3-Ab stimulated without MW167 ($p=0.01$). The addition of anti-CD28 stimulation did not restore IL-10 secretion in the presence of MW167, with a mean decrease of 83±5.8% from anti-CD3/28-Ab treated cells ($p=0.009$) (Figure 4.7A).

The same trend was observed in groups cultured for 72 hours, with MW167 treatment reducing IL-10 secretion by 69.6±8.3% ($p=0.001$) by anti-CD3-Ab stimulated cells (Figure 4.7B). Activation with anti-CD3/28-Ab did not over-come inhibition of IL-10 secretion by MW167, down 58.4±19% ($p=0.011$) in MW167 treated groups compared to control cells (Figure 4.7B).

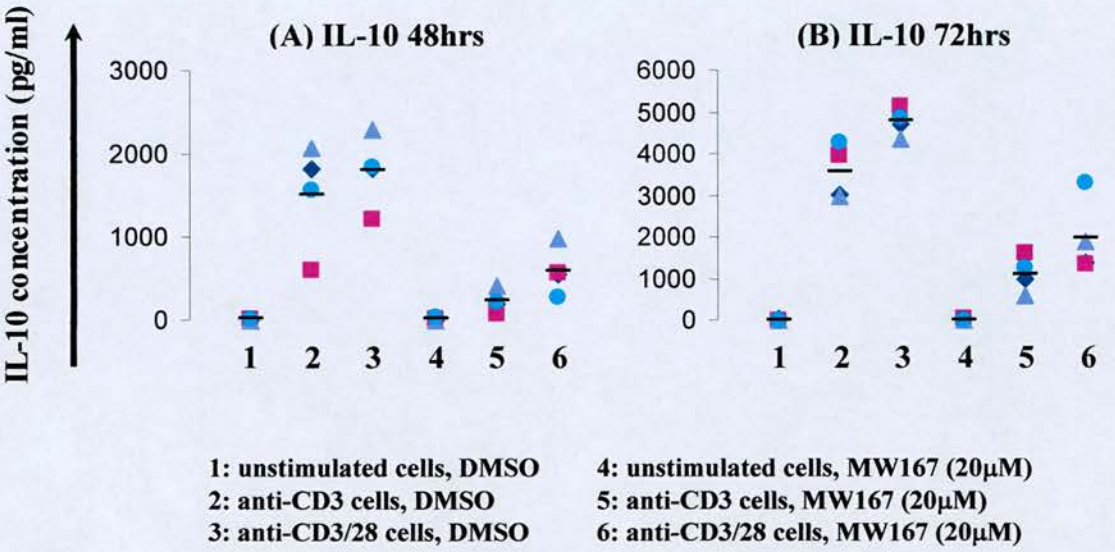


Figure 4.7: IL-10 secretion by anti-CD3- and anti-CD3/28-Ab stimulated $CD4^+$ T cells is inhibited in the absence of Notch signalling. IL-10 secretion by unstimulated, anti-CD3- or anti-CD3/28-Ab activated $CD4^+$ T cells cultured for 48 hours (A) and 72 hours (B) in the absence or presence of MW167. Supernatants were assayed by ELISA. Four individual experiments are each represented as one colour of icon; the mean value is depicted as a black bar.

Real-time RT-PCR primers and probes were available for examination of IL-10 gene transcription. Total RNA extracted from unstimulated, anti-CD3- and anti-CD3/28-Ab treated cells in the absence or presence of MW167 after 48 hour culture. Data is presented as expression relative to the unstimulated control cell group (cells plus DMSO carrier). Four RNA sets were collected for this experiment. Unfortunately, two sets were of too poor quality to yield interpretable results from the real-time RT-PCR reaction.

IL-10 transcription was induced upon $CD4^+$ T cell stimulation with anti-CD3-Ab alone. In one experiment, addition of anti-CD28-Ab to CD3 stimulation augmented

transcription of IL-10. In both experiments, IL-10 transcription was inhibited in the presence of 20μM MW167 when cells were stimulated with anti-CD3, with fold decreases of 96.7% and 89.1% compared to control groups (Figure 4.8A and B respectfully). Inhibition of transcription was also observed when cells were stimulated with anti-CD3/28-Ab in the presence of MW167 compared to stimulated cells without γ-secretase inhibitor demonstrating fold decreases of 89% and 82.9% (Figure 4.8A and B).

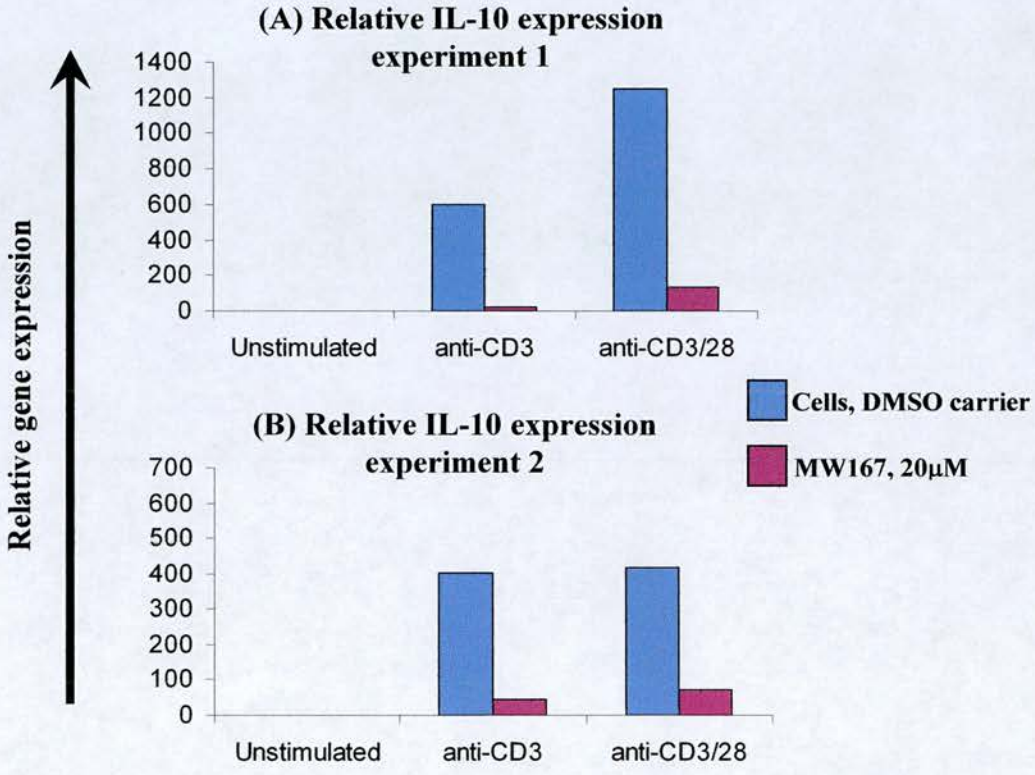


Figure 4.8: Transcription of IL-10 is inhibited in the absence of Notch signalling. IL-10 gene expression by unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells was assessed by real-time RT-PCR. Expression is presented relative to unstimulated cells cultured with DMSO carrier. Data from two independent experiments are represented as figure A and B respectively.

Having established that IL-10 gene transcription was attenuated in the absence of Notch signalling, it was hypothesised that components of the Notch pathway may interact directly with the IL-10 promoter. Examination of the IL-10 promoter (Genbank accession: M84340) using the Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/tess/) revealed a potential murine HES1 binding site (1594-9), the relevance of which remains to be ascertained. Blasting this mouse sequence against the human IL-10 promoter sequence (Genbank accession: AF295024) to identify homologous regions revealed that the potential murine HES1 binding site was conserved between the two sequences (Figure 4.9). No putative RBP-Jκ binding sites were identified.

		HES1 binding site	
Mouse IL-10:	1570	gacttgctcttgcactaccaaagc**cacaag**gcagccttgca	1610
Human IL-10:	726	ggcttgctcttgcaaaaccaaa-c**cacaag**acagacttgca	765

Figure 4.9: Identification of potential murine HES1 binding site in the IL-10 promoter. TESS revealed a potential murine HES1 binding site in the mouse IL-10 gene sequence. Alignment with human IL-10 sequence revealed this region was conserved.

4.7 NFAT activity is unaffected by inhibition of Notch signalling

MW167 inhibition of cytokine secretion by anti-CD3-Ab stimulated $CD4^+$ T cells suggested that endogenous Notch signalling promotes cytokine secretion when co-stimulation is limiting. The finding that blockade of γ–secretase activity inhibits IL-10 transcription, and documented interactions of Notch with numerous transcriptional regulators, the effect of MW167 treatment on TCR targeted transcription factors was examined. Classically, TCR signalling activates transcriptional activity of NFAT, NFκB and AP-1(Jun/Fos). This section of work aimed to examine NFAT activity, time constraints prevented follow-up of NFκB and Ap-1 activity.

A DO11.10 hybridoma cell line stably transfected with GFP under the control of an NFAT regulated promoter (108) (kindly gifted by Dr James Brewer, University of Glasgow) was used to determine the effect of MW167 on NFAT activity. A preliminary test for expression of *hes1* and *IL-10* in the absence or presence of MW167 was carried out before progression to analysis of NFAT activity. As with primary murine $CD4^+$ T cells, relative *hes1* expression increased upon stimulation with anti-CD3-Ab compared to unstimulated cells (Figure 4.10A) by real-time RT-PCR. Incubation with 20μM MW167 reduced *hes1* expression in unstimulated hybridoma cells and prevented up-regulation upon CD3 ligation (Figure 4.10A). Gene expression of IL-10 was also assayed, again revealing transcriptional repression when γ-secretase activity was inhibited (Figure 4.10B).

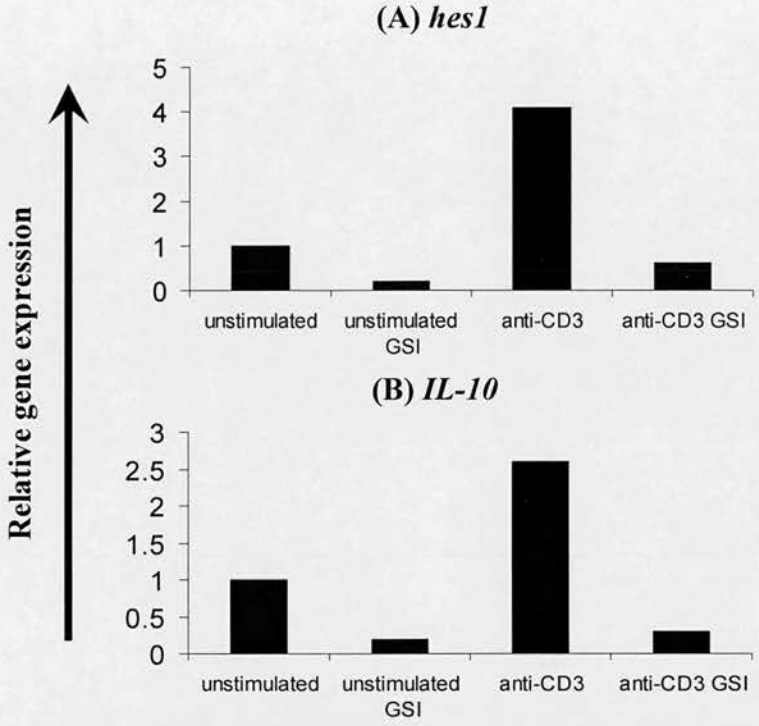


Figure 4.10: Inhibition of *hes1* and *IL-10* transcription in NFAT-GFP DO11.10 hybridoma by MW167. Real-time RT-PCR was used to confirm reduced *hes1* and *IL-10* transcription in DO11.10 hybridoma cells in the presence of MW167 at 20μM (GSI: γ-secretase inhibitor). Cells were incubated in the absence or presence of MW167 for 4 hours, then cultured for a further 8 hours as unstimulated or anti-CD3-Ab treated cells.

As NFAT activity is quickly induced after TCR ligation, cells were incubated for 8 hours post DMSO carrier or MW167 incubation, as unstimulated or anti-CD3-Ab treated cells. Anti-CD3/28-Ab treated groups were not included in this series of experiments as the aim was to determine a link between TCR signalling and Notch activity. Additionally, many T cell hybridomas appear to be refractory to co-stimulation. GFP expression was analysed by flow cytometry (Figure 4.11), a viable cell gate was used based on forward and side scatter profiles (Figure 4.11A, B).

GFP expression was not evident in unstimulated cultures (Figure 4.11C, E), consistent with the requirement for TCR engagement for activation of this reporter

construct. Hybridoma treatment with MW167 did not affect GFP expression and therefore NFAT activity in unstimulated cells (Figure 4.11C, E). Hybridoma stimulation with anti-CD3-Ab induced GFP expression in both the absence and presence of MW167 (Figure 4.11D, F). No discernable difference was evident in percentage of GFP positive cells or the mean fluorescent intensity between DMSO carrier and MW167 treated groups (Figure 4.11D, E).

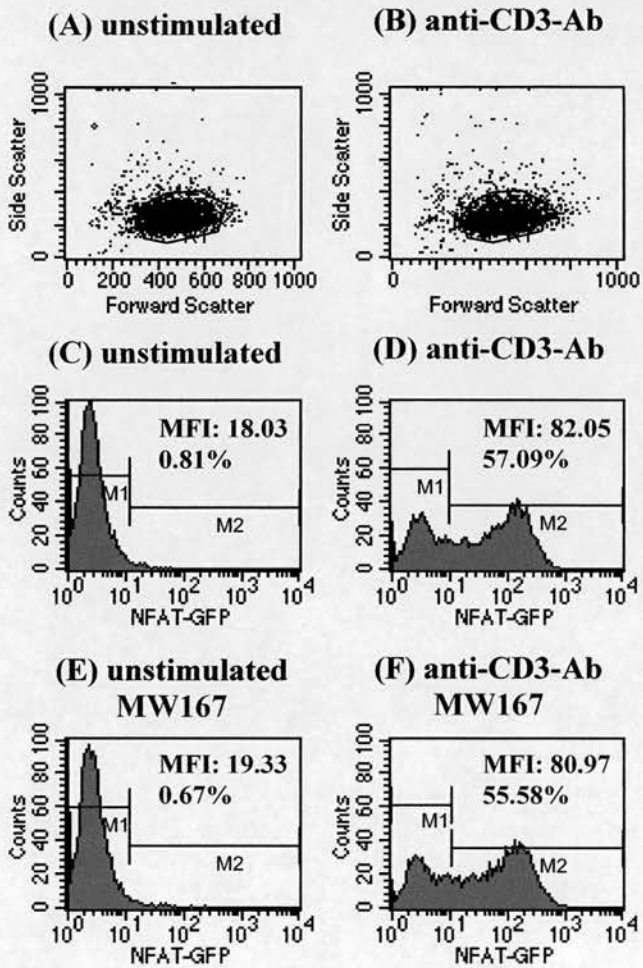


Figure 4.11: NFAT activity is unaffected by MW167. NFAT-GFP DO11.10 hybridoma stable transfectants were incubated with carrier DMSO or MW167 (20μM) for 4 hours then cultured for a further 8 hours as unstimulated or anti-CD3-Ab treated cells. Cells were harvested and GFP expression assessed by flow cytometry. A-B show forward and side scatter profiles of unstimulated and anti-CD3-Ab treated hybridoma cells respectively. Histogram plots show expression of GFP by unstimulated (C) and anti-CD3-Ab stimulated cells (D) in the absence of MW167. Panels (E) and (F) depict unstimulated and anti-CD3-Ab treated cells in the presence of 20μM MW167. Percentages and mean fluorescent intensities (MFI) for M2 are shown. Data is representative of three experiments.

4.8 Discussion

Inhibition of the γ-secretase complex has revealed that Notch signalling is required for TCR induced TNFα, IFNγ, IL-4 and IL-5 secretion in the absence of CD28 co-stimulation in $CD4^+$ T cells. Secretion of IL-10 appears to be dependent on reception of Notch signalling and TCR ligation even in the presence of co-stimulation. Notch inhibition did not affect cell viability, nor did it alter the proliferative response.

Treatment of unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells with the γ-secretase inhibitor MW167 inhibited *hes1* transcription in a dose dependent manner. Use of MW167 at 20μM yielded the most consistent inhibition of Notch signalling, as measured by *hes1* expression. This dosage was found to be in keeping with that used in developmental studies, where inhibition of Notch was measured directly by quantification of NICD generation (67). γ-secretase inhibition prevented up-regulation of *hes1* upon TCR stimulation confirming that T cell receptor signalling does not directly induce *hes1* expression, validating the use of *hes1* transcription as a read-out of Notch activity. Since undertaking this section of work, two independent studies have employed γ-secretase inhibitors to interrupt Notch signalling in thymocytes (149, 150), demonstrating similar effects on thymocyte development to those observed in conditional Notch knockout mice. The use of γ-secretase inhibitors to influence peripheral T cell activation was also recently documented, further confirming the validity of γ-secretase inhibition as a means of Notch signal disruption in lymphoid cells (97, 96).

Disruption of Notch signalling did not affect cell viability, ruling out possible toxicity effects of the inhibitor or that increased cell death had influenced detection of *hes1* transcripts. This demonstrated that unstimulated $CD4^+$ T cells, anti-CD3- and anti-CD3/28-Ab treated cells lacking Notch signalling were no more susceptible to death in culture than control cells. This is not to say that induction of Notch signalling is not anti-apoptotic, as seen in thymocytes, where Notch can rescue cells from glucocorticoid induced cell death (119).

Attenuation of Notch signalling in mature T cells has been shown to be detrimental to activation induced proliferation (97, 96). Use of γ-secretase inhibitors, Notch1 anti-sense transgenic mice (Notch AS) and retroviral Notch transduction have recently linked this pathway with $CD4^+$ T cell expansion (97, 96). γ-secretase inhibition limits secretion of IL-2, potentially limiting T cell proliferation (97). CD25 expression was reduced on addition of γ-secretase inhibitor while over-expression of Notch1 enhanced proliferative responses (97). However, treatment of unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells with 20μM MW167 did not affect proliferation. This discrepancy is most likely due to the different pharmacological agents used and possibly to the strength of TCR signals delivered (different sources and quantities of monoclonal antibodies having been used). Although the γ-secretase inhibitor, compound E, inhibited proliferation to 0.1μg/ml OVAp, it had no effect when 1μg/ml OVAp was used, demonstrating that the strength of TCR signalling influences the effect of Notch. In the data set presented here, purified $CD4^+$ T cells are used whereas the studies using compound E and IL-CHO γ-secretase inhibitors look at T cell responses as part of mononuclear or whole

spleen populations (97, 96). This raises the possibility that γ-secretase inhibitors affect APCs, potentially limiting their ability to induce T cell proliferation and cytokine secretion. Proliferation of Notch1 AS splenocytes in response to anti-CD3-Ab stimulation was normal, indicating that Notch1 does not influence T cell expansion (96). This indicates the likely difference in function between Notch family members, but may also relate to the role of other γ-secretase substrates influencing lymphocyte proliferation. APP is known to be expressed by immune system cells (151) and has been implicated in augmenting T cell proliferation (152).

Anti-CD3-Ab induced cytokine secretion by $CD4^+$ T cells was inhibited in the presence of MW167, indicating that Notch signalling is required for release of TNFα, IFNγ, IL-4 and IL-5. There did not seem to be preferential inhibition of Th1 or Th2 cytokine secretion by γ-secretase inhibitor. The inability of MW167 to inhibit cytokine secretion by anti-CD3/28-Ab stimulated cells suggests that Notch may function as a co-stimulator and may reflect parallel activation of analogous signalling cascades by Notch and TCR/CD28, as Notch signalling can regulate both NFκB and Ras activity (88, 134). IFNγ secretion by IL-CHO treated, and Notch1 AS transgenic mouse anti-CD3-Ab-stimulated splenocytes was found to be significantly reduced (96), confirming our own findings. In the aforementioned study, IFNγ secretion was not restored when CD28 signalling was present in γ-secretase inhibitor treated, activated cultures (96). This disparity is possibly due to different co-stimulatory signals present in whole splenocyte populations and possible γ-secretase inhibitor effects on APCs, compared to the study of purified $CD4^+$ T cells.

NFAT function has been described in the regulation of numerous genes pertaining to productive immune responses (153). NFAT interacts with IL-2, IFNγ and IL-4 promoters, acting alone, with AP-1 or, as in the IL-4 promoter, with JunB (154). The absence of IFNγ and IL-4 secretion by $CD4^+$ T cells stimulated with anti-CD3-Ab in the absence of Notch signalling may have been due to reduced activity of NFAT activity. To assess this possibility, NFAT activity was measured using a DO11.10 hybridoma cell line stably transfected with an NFAT-GFP reporter. The hybridoma was shown to respond similarly to primary murine $CD4^+$ T cells in that treatment with MW167 inhibited transcription of *hes1* and *IL-10*. Flow analysis of GFP expression did not reveal any alteration in NFAT activity by unstimulated or anti-CD3 treated hybridoma cells when incubated in the presence of the γ-secretase inhibitor. This finding was corroborated by a recent publication demonstrating no alteration in NFAT activity in splenocyte populations treated with the γ-secretase inhibitor IL-CHO (96). Although only NFAT data is presented in this thesis, the prime candidate for mediating the observed Notch effect on cytokine secretion was NFκB.

NFκB proteins consist of homo- or heterodimers of Rel family proteins (155). T cell activation releases inhibition by IκB and allows DNA binding by NFκB. Many genes are induced on Rel family binding, regulating proliferation, apoptosis and cytokine production (156). Developmental studies have previously related NFκB as determining and Notch as inhibiting cell fate (157), however this appears context dependent and in some instances both pathways may work synergistically. It appears Notch regulates NFκB family transcription factors both negatively and positively.

NICD is able to bind NFκB in a mechanism similar to IκB, inhibiting its nuclear activity (138, 158). A variation on this is the finding that p65/IκBα shuttles the co-repressors N-CoR and SMRT to the cytoplasm, enhancing *hes1* transcription (146). The authors of this paper propose an intriguing interaction between the two pathways, with absence of NFκB activation augmenting Notch signalling, where p65/IκBα associates with transcriptional co-repressors. Degradation of IκK releases repression on NFκB and allowing κB gene transcription and re-association of CBF-1/NICD/co-repressor complexes. This mechanism does not correlate with our findings, where activation of NFκB activity by Notch would relate to co-stimulatory properties. In contrast, introduction of Notch3ICD under the control of the proximal *lck* promoter results in increased NFκB activity in thymocytes, with these mice going on to develop T-lymphoblastic lymphomas (88). The idea that Notch signalling promotes NFκB signalling is consistent with a role for Notch in augmenting T cell activation. Anti-CD3/28-Ab activation of γ-secretase inhibitor treated or Notch1 AS splenocytes was associated with reduced NFκB activity, and was a likely cause of the reduced IFNγ secretion observed (96). The finding that addition of CD28 co-stimulation recovered TNFα, IFNγ, IL-4 and IL-5 secretion supports the hypothesis that Notch may facilitate cytokine production by increasing NFκB activity. NFκB activity is reported to be a pertinent target of CD28 co-stimulation. CD28 assists TCR in activation of the IKK complex via MAP3Ks, PKCθ and Akt, mediating release of NFκB repression (159).

The observation that Notch signalling is required for $CD4^+$ T cell secretion of IL-10 in the absence or presence of CD28 co-stimulation alludes to a role in tolerance/regulation. IL-10 is important in the generation and/or function of various regulatory T cell populations, in particular Tr1 cells (50, 160, 161). Human $CD25^+CD4^+$ T cells express high levels of *notch4* and *delta1* transcripts (98) and the correlation between Notch signalling and IL-10 could suggest a mechanism for $CD25^+CD4^+$ T reg induction of Tr1 cells. Indeed, over-expression of the Notch ligand Jagged1 by APCs induces a population of regulatory T cells (1). The mechanism by which these T regs mediate suppression is unclear, but it is possible that Notch signalling does not induce a T reg phenotype directly but does so by promoting IL-10 secretion. It has been proposed that Notch signalling, induced on ligation by a Delta1 fusion protein, stimulates IL-10 production (107). While findings presented in this thesis support this hypothesis, the Delta1 fusion protein has been reported to inhibit inflammatory cytokine secretion (107). This difference may be due to suppression of endogenous signalling, as opposed to over-induction of Notch pathway activation.

IL-10 transcription is independent of NFκB activity, demonstrated by studies using chemical inhibition or IkB over-expression (162, 163). Indeed, IL-10 transcriptional control is different from that of pro-inflammatory cytokines as it lacks functional binding sites for transcription factors such as C/EBP and AP-1 (162). The deficiency of these transcription factor-binding sites may partly explain our observation that addition of anti-CD28-Ab did not restore IL-10 secretion in the absence of Notch. Examination of the IL-10 promoter (Genbank accession: M84340) using TESS

revealed a potential HES1 binding site (1594-9), the relevance of which remains to be ascertained. Further biochemical analysis of Notch signal transduction and its interaction with TCR and co-stimulatory signals is required.

4.9 Future work

The use of γ-secretase inhibitors to block Notch signalling in T cells was recently demonstrated to attenuate proliferative responses (97, 96). However, γ-secretase inhibitors can block cleavage of the amyloid precursor protein (164). Addition of APP peptides upon stimulation of mononuclear cells with anti-CD3 was observed to enhance proliferative response (152). APP expression is also observed to be induced upon T cell activation (151). The γ-secretase inhibitor used throughout this PhD, MW167, did not inhibit $CD4^+$ T cell proliferation in response to polyclonal activation. In an attempt to reconcile this difference between use of MW167 and those in recent publications (Compound E and IL-CHO) (97, 97), a preliminary experiment was carried out using a γ-secretase inhibitor specific for APP (γ-secretase inhibitor XI) and Compound E. Inhibition of APP cleavage in anti-CD3-Ab and anti-CD3/28-Ab stimulated $CD4^+$ T cell cultures prevented proliferation, possibly inducing cell death. Compound E marginally reduced proliferation but not to the same degree as reported in the literature. MW167 was also used in culture with anti-CD3-Ab stimulated whole spleen (using the 100μM dosage) and did not affect proliferation. These preliminary findings suggest that care should still be taken when considering a role for Notch in T cell proliferative responses.

Examination of surface marker expression by flow cytometry would be worth pursuing given evidence that CD69 and CD25 levels are influenced by Notch signalling (93, 97). This was addressed by another PhD student in the group (Dr Karen Adamson) in conjunction with the experiments presented in this chapter and suggested expression of neither CD25 or CD69 was affected by MW167. However, this is only one γ-secretase inhibitor and should be assessed in the context of others.

Gel shift and super shift assays would be the best method for further determining whether HES1 or CBF-1 proteins are capable of binding directly to the IL-10 promoter. Attempts were made to achieve this, however, this aspect was abandoned due to a lack of time and technical ability! All reagents are in place to pursue this avenue and would prove informative.

Generation of the NFκB-GFP hybridoma cell line, use of Ras/Erk phosphorylation ELISAs and cytometric bed analysis of Akt activity would have been the major avenues pursued in dissecting the interaction between Notch and TCR/costimulatory signalling.

Finally, restimulation of $CD4^+$ T cells that were originally primed in the presence of γ-secretase inhibitors would have established if a lack of Notch signalling on initial activation results in a terminal effect upon differentiation.

5 Notch and regulatory $CD4^+$ T cells

5.1 Introduction/background

Active regulation/suppression by $CD4^+$ T cells has become the focus of many tolerance models, encompassing responses to both self and foreign antigen (165). Several types of $CD4^+$ T regulatory cells have been described and can be generalised as being $CD25^+CD4^+$ T regs, Tr1 or Th3 (166, 167). These may exist as naturally occurring or antigen induced populations (24). Increasing evidence implicates a role for Notch signalling in the adoption of T regulatory phenotypes by $CD4^+$ T cells.

Direct evidence of Notch signalling mediating tolerance comes from two studies where Notch ligands were over-expressed on APCs. Stimulation of naïve $CD4^+$ T cells *in vivo* with antigen pulsed DCs over-expressing Serrate1 (Jagged1) prevented induction of protective immunity (1). Tolerance could also be transferred by the $CD4^+$ T cell compartment, implicating active regulation. Unfortunately the population of regulatory cells generated in this model were not characterised for their ability to proliferate or secrete cytokines. A more recent study utilising a mismatched haplotype system revealed that over-expression of Jagged1 on APCs induced differentiation of a Th3 like regulatory population, further supporting a role for Notch in mediating tolerance (105).

Tr1 cell function and generation is heavily dependent on the immunoregulatory cytokine IL-10 (168). In Chapter 4 of this thesis (Chapter 4.6, page 119), Notch signalling was linked to the secretion of IL-10 by $CD4^+$ T cells. Evidence in support of this pertains to the function of the Notch ligand Delta-like1. Incubation of anti-CD3/28-Ab activated $CD4^+$ T cells with an Fc-Delta-like1 fusion protein is reported to enhance IL-10 secretion and inhibits secretion of inflammatory cytokines (107). It has been speculated that cells treated in this fashion function like Tr1 cells (107).

It has been hypothesised that $CD25^+CD4^+$ T regs are generated naturally in the thymus through a process of "altered negative selection". In this model, cells expressing an αβ TCR with a relatively high affinity for self antigen/MHC class II are not deleted, but exit the thymus as regulators of self (47). Progression of thymocyte development is highly dependent on the Notch signalling pathway (169). Notch signalling mediates commitment to the T lineage, but also regulates αβ versus γδ TCR expression, promotes progression to the $CD4^+CD8^+$ double positive thymocyte stage and may influence commitment to CD4 or CD8 single positive cells (169). It might be speculated that Notch may also be involved in the generation of $CD25^+CD4^+$ T cells as they undergo selective pressures in the thymus. In line with this idea is the demonstration that NICD over-expression in a T cell hybridoma inhibited Nur77 dependent apoptosis, relevant for TCR induced cell death upon negative selection (102). Additionally, since Notch regulates CD25 expression in T cells (97, 170), could it also be involved in inducing constitutive expression of this molecule in $CD25^+CD4^+$ T regs?

Chapter aims:

- Purify $CD25^{+}CD4^{+}$ T cells from murine spleens;
- Demonstrate that these cells are capable of regulating $CD4^{+}$ T cell proliferation;
- Assess relative expression of Notch pathway components in $CD25^{+}$ compared to $CD4^{+}CD25^{-}$ T cells;
- Determine a role for Notch in mediating regulation by $CD25^{+}CD4^{+}$ T cells;
- Generate Tr1 cells *in vitro*;
- Demonstrate regulatory capacity of Tr1 cells;
- Assess relative expression of Notch pathway components in Tr1 cells compared to non regulatory $CD4^{+}$ T cells.

5.2 Regulation by murine $CD25^+CD4^+$ T cells

$CD25^+CD4^+$ T cells were purified from BALB/c spleens using the Miltenyi purification kit in conjunction with the autoMACs (Chapter 2.2, page 66). Release of this optimised kit by Miltenyi overcame problems encountered by previous attempts to purify these cells using various combinations of magnetic beads and FACs sorting. An enriched population, 89.7±1.6% (n=6) $CD25^+CD4^+$ (Figure 5.1A), was used obtained. Proliferative responses of unstimulated, anti-CD3- and anti-CD3/28-Ab stimulated $CD25^+CD4^+$, $CD4^+CD25^-$ and co-cultures of these two populations were compared to determine whether the $CD25^+$ population could affect proliferation of $CD25^-$ T cells (Figure 5.1).

$CD4^+CD25^-$ T cells were seeded at $5x10^4$ cells per well of 96 well plates, in ratios of 0:1, 1:2 and 1:1, $CD25^+CD4^+$: $CD4^+CD25^-$. Control cultures of 10^5 $CD4^+CD25^-$, the maximum number of T cells used in any well, were included to ensure reduced proliferation in co-cultures was not due to nutrient depletion. Data from three individual experiments is presented.

Unstimulated cells did not proliferate to any great degree over the 72 hour culture period, as might be expected. However, at the maximal seeding density of 10^5 T cells per well, $CD4^+CD25^-$ T cells consistently incorporated more [^{3}H] thymidine than $CD25^+CD4^+$ T cells ($p=0.005$) (Figure 5.1B). $CD25^+CD4^+$ T cells were significantly hypo-responsive to anti-CD3-Ab stimulation in comparison to $CD4^+CD25^-$ T cells

(p=0.000003, n=3) (Figure 5.1C). Stimulation of cells with anti-CD3/28-Ab enhanced proliferation of both cell subsets relative to anti-CD3-Ab alone (p=0.0004 for $CD25^+CD4^+$ and p=0.003 for $CD4^+CD25^-$) (Figure 5.1 C and D). A marked difference between $CD25^+$ and $CD4^+CD25^-$ cell proliferation upon anti-CD3/28-Ab stimulation was also observed, further demonstrating $CD25^+$ T cell hypo-responsiveness (p=0.00005) (Figure 5.1D). Regulation of $CD4^+CD25^-$ T cell proliferation by $CD25^+CD4^+$ T cells was not observed in unstimulated, anti-CD3- or anti-CD3/28-Ab treated cultures (Figure 5.1B, C and D respectfully). Thus $CD25^+CD4^+$ T cells are hyporesponsive and are unable to regulate $CD25^-$ T cells proliferation in response to stimulation with anti-CD3- or anti-CD3/28-Ab. Lack of regulation in these cultures was not unexpected given the body of evidence indicating a role for APCs in suppression by murine $CD25^+CD4^+$ T cells. However, this was the first time these cells had been used in the group and as such full characterisation was required to ensure that the purified cells were the same as those detailed in published literature.

Co-cultures incorporating APCs were used to demonstrate active regulation. The cultures utilised the same numbers of T cells as described above but with the addition of mitomycin C treated splenocytes as APCs and anti-CD3-Ab. Three individual experiments were carried out, assessing proliferation by $[^3H]$ thymidine incorporation, harvesting cultures after 72 hours (Figure 5.2). Proliferation by $CD25^+CD4^+$ T cells was minimal (Figure 5.2A). Co-culture of $CD4^+CD25^-$ T cells with $CD25^+CD4^+$ T cells using anti-CD3-Ab/APCs as the activation stimulus allowed regulation to occur. $CD4^+CD25^-$ T cell proliferation was reduced by

70.9±7% in the presence of $CD25^+CD4^+$ T cells at a ratio of 1 $CD25^+$:2 $CD4^+CD25^-$ (p=0.00013) (Figure 5.2A). $CD4^+CD25^-$ T cell proliferation could not be reduced further by increasing the co-culture ratio to 1:1, with proliferation having been reduced by 81.3±3% (p=0.00002) (Figure 5.2A).

Analysis of supernatants from $CD25^+CD4^+$, $CD4^+CD25^-$ and co-cultures activated with anti-CD3-Ab/APCs revealed that secretion of TNFα, IFNγ, IL-4, IL-5 and IL-10 (Figure 5.2B, C, D, E and F respectfully) mirrored proliferation data, in that groups having low [^{3}H] thymidine incorporation also secreted low concentrations of cytokines. Low levels of TNFα were detected in $CD25^+CD4^+$/APC anti-CD3-Ab stimulated cells (Figure 5.2B); IFNγ secretion was undetectable (Figure 5.2C). Low levels of IL-4 and IL-5 were detectable in $CD25^+CD4^+$ groups (Figure 5.2D, E). $CD4^+CD25^-$ T cells cultures secreted considerably higher levels of cytokines than $CD25^+CD4^+$ cells. Co-culture of cells reduced cytokine secretion to levels equivalent to mitomycin C treated splenocytes on their own (Figure 5.2). IL-10 secretion in 1:2 and 1:1 cultures remained relatively high given the absence of proliferation in these groups (Figure 5.2F).

T cells incubated with mitomycin C treated splenocytes without anti-CD3-Ab did not incorporate [^{3}H] thymidine above the counts observed for splenocytes alone. Cytokine secretion in these unstimulated cultures was below the limit of detection for the assays used.

$CD25^+CD4^+$ T cell hypo-responsiveness and inhibition of $CD4^+CD25^-$ T cell proliferation to anti-CD3-Ab/APC stimulation was visually evident, with smaller and less clumps of cells forming, reflecting poor cell expansion (Figure 5.3).

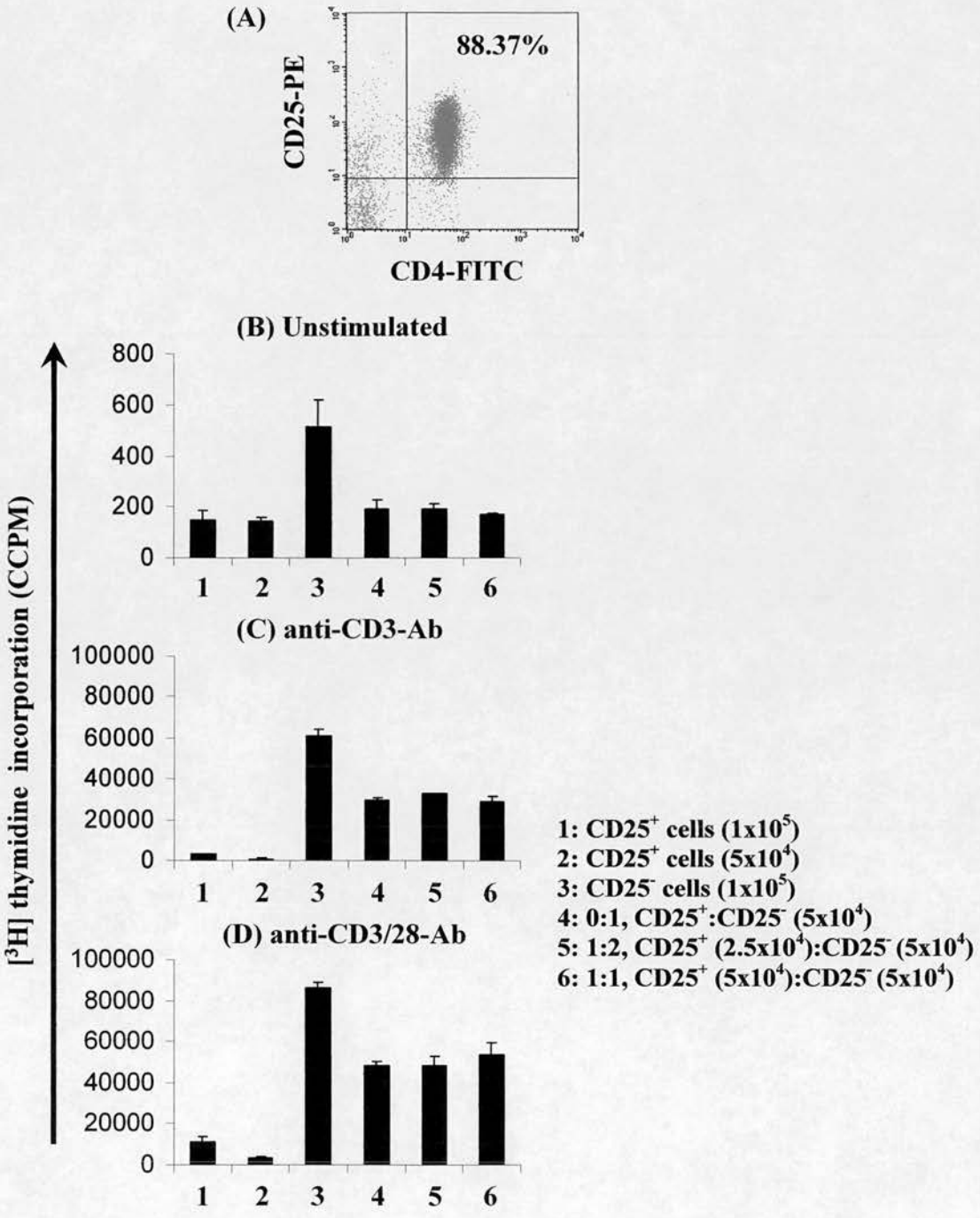


Figure 5.1: Isolation of $CD25^+$ $CD4^+$ T cells hyporesponsive to TCR stimulation. $CD25^+$ $CD4^+$ T cells were purified by autoMACs separation using the murine $CD25^+CD4^+$ T cell purification kit (A). Proliferation of unstimulated (B), anti-CD3-Ab (C) and anti-CD3/28-Ab (D) treated $CD25^+$, $CD25^-$ $CD4^+$ and co-cultured $CD25^+/CD25^-$ $CD4^+$ T cells was assessed by $[^3H]$ thymidine incorporation. Cells were harvested at 48 hours. One representative experiment of four is shown.

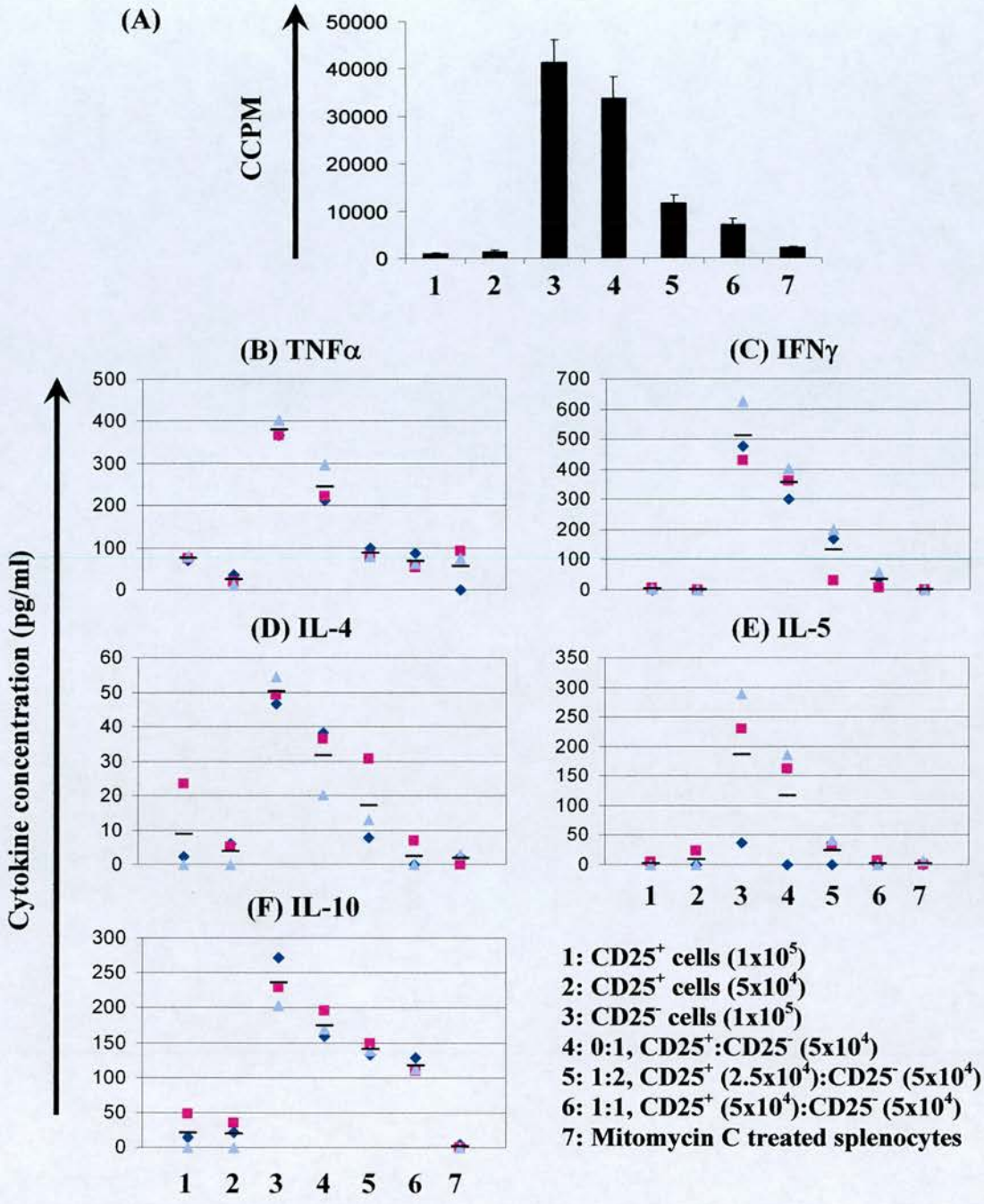


Figure 5.2: Regulation of $CD4^+CD25^-$ T cell proliferation and cytokine secretion by $CD25^+CD4^+$ T cells. $CD25^+$ and $CD25^-$ $CD4^+$ T cells were MACs purified and cultured with $CD4^+$ cell depleted mitomycin C treated anti-CD3-Ab pulsed splenocytes. Proliferation was measured at 48 hours by [^{3}H] thymidine incorporation, one representative experiment of three is shown (A). Secretion of TNFα, IFNγ, IL-4 and IL-5 was measured by cytometric bead analysis (B, C, D and E respectfully). IL-10 secretion was assessed by ELISA (F). Supernatants were harvested at 48 hours. Three individual experiments are each represented by as one colour of icon; the average value is depicted as a black bar.

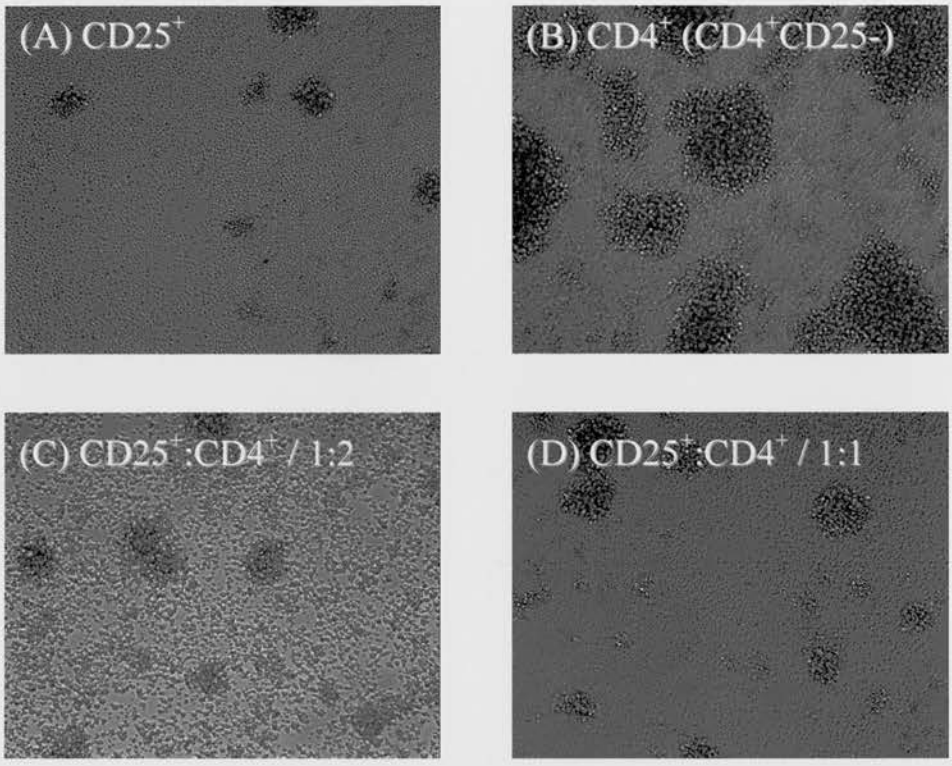


Figure 5.3: $CD25^+CD4^+$ T cell suppression of $CD4^+CD25^-$ T cell expansion. $CD25^+CD4^+$ T cells were MACs purified and stimulated with anti-CD3-Ab pulsed, mitomycin C treated splenocytes (A). $CD4^+$ T cells depleted of $CD25^+$ cells were stimulated in the same way (B). $CD25^+CD4^+$ T cells were added in increasing numbers to the $CD4^+CD25^-$ cultures (C, D), either $2.5x10^5$ $CD25^+$ cells (C) or $5x10^5$ (D).

5.3 Notch gene expression by $CD25^+CD4^+$ T cells

Human $CD25^+CD4^+$ T cells are reported to express elevated levels of *notch4*, *hes1*, *deltex* and *delta-like1* (98). This prompted further investigation of expression of Notch components in $CD25^+CD4^+$ T cells. Cells were purified as previously described (Chapter 2.2, page 66) and incubated with mitomycin C treated splenocytes, in the presence or absence of anti-CD3-Ab. Cells were harvested at 48 hours and total RNA extracted. Relative expression of *hes1*, *deltex*, *notch1*, *2*, *3*, *4*, *jagged1*, *2* and *delta-like1* was then assessed by real-time RT-PCR (Figure 5.4). Expression is plotted as a relative unit compared to unstimulated $CD4^+CD25^-$ T cells. Relative fold changes of 2 or greater are considered biologically relevant, relating to relative gene expression values of 2 or 0.5.

No difference in expression of *hes1* expression between $CD25^+CD4^+$ and $CD4^+CD25^-$ T cells was observed (Figure 5.4A). Activation induced a slight increase in *hes1* expression by $CD25^+CD4^+$ T cells, mean relative expression of 2.2±0.9 (Figure 5.4A). Activation of $CD4^+CD25^-$ with anti-CD3-Ab/APCs induced a higher relative *hes1* increase to 7.98±1 compared to unstimulated cells (Figure 5.4A). Relative deltex expression by unstimulated $CD25^+CD4^+$ T cells was comparable to unstimulated $CD4^+CD25^-$ T cells (Figure 5.4B). Activation of both $CD25^+CD4^+$ and $CD4^+CD25^-$ T cells resulted in decreased *deltex* expression, with mean relative expression of 0.06±0.04 and 0.11±0.03 respectively (Figure 5.4B).

No biologically relevant difference in *notch1*, *2*, *3*, or *4* gene expression was evident between unstimulated $CD25^+CD4^+$ and $CD4^+CD25^-$ T cells (Figure 5.4C, D, E and F). Activation induced a trend for increased *notch1* expression by $CD4^+CD25^-$ T cells, with a 2.78±3 relative fold increase being detected (Figure 5.4C). Activation did not induce any biologically relevant change in *notch1* expression by $CD25^+CD4^+$ T cells (Figure 5.4C). Activation reduced expression of *notch2* by $CD25^+CD4^+$ T cells, mean relative expression 0.24±0.04, but not by $CD4^+CD25^-$ T cells, mean relative expression 1.09±0.2 (Figure 5.4D). A trend for reduced *notch3* expression was observed for both $CD25^+CD4^+$ T cells and $CD4^+CD25^-$ upon activation (Figure 5.4E). Reduction in relative *notch3* expression by anti-CD3-Ab/APC stimulated $CD25^+CD4^+$ T cells was biologically relevant, falling to a value of 0.24±0.09 (Figure 5.4E). Mean relative expression of *notch3* by stimulated $CD4^+CD25^-$ T cells was not deemed biologically relevant, 0.45±0.3 (Figure 5.4E). This may not be a true reflection of the data, with two out of three results having relative expression values of 0.4, an increased number of replicates are required for clarification. Anti-CD3-Ab/APC stimulation of $CD25^+CD4^+$ T cells did not reduce relative *notch4* expression to any great extent, mean relative expression 0.77±0.04 (Figure 5.4F). Mean relative expression by stimulated $CD4^+CD25^-$ T cells was 0.3±0.23 (Figure 5.4F), bordering on biological relevance, again requiring increased numbers of replicates to clarify this data.

Differences in *jagged1* and *2* mean relative expression between unstimulated $CD25^+CD4^+$ and $CD4^+CD25^-$ T cells was minimal, never reaching biological

relevance (Figure 5.4G, H). Activation of $CD25^+CD4^+$ T cells did not reduce *jagged1* expression but did decrease $CD4^+CD25^-$ T cell mean relative expression to 0.33±0.03 (Figure 5.4G). This equates to an approximate 3.7 fold difference in *jagged1* expression between activated $CD25^+$ and $CD25^-$ T cells. Relative expression of *jagged2* by $CD25^+CD4^+$ and $CD4^+CD25^-$ T cells was reduced upon stimulation, mean relative expression falling to 0.2±0.09 and 0.37±0.05 respectfully (Figure 5.4H).

Relative expression of *delta-like1* by unstimulated $CD25^+CD4^+$ cells was found to be 10.9±2.2 fold higher than unstimulated $CD4^+CD25^-$ T cells (Figure 5.4I). $CD25^+CD4^+$ T cell expression was decreased upon anti-CD3-Ab/APC stimulation, falling to a mean relative value of 2.34±0.22. Relative expression by stimulated $CD4^+CD25^-$ T cells was also reduced, 0.19±0.02 (Figure 5.4I). This relates to a fold difference of 12.23±2.3 in *delta-like1* relative expression between activated $CD25^+$ and $CD25^-$ cells.

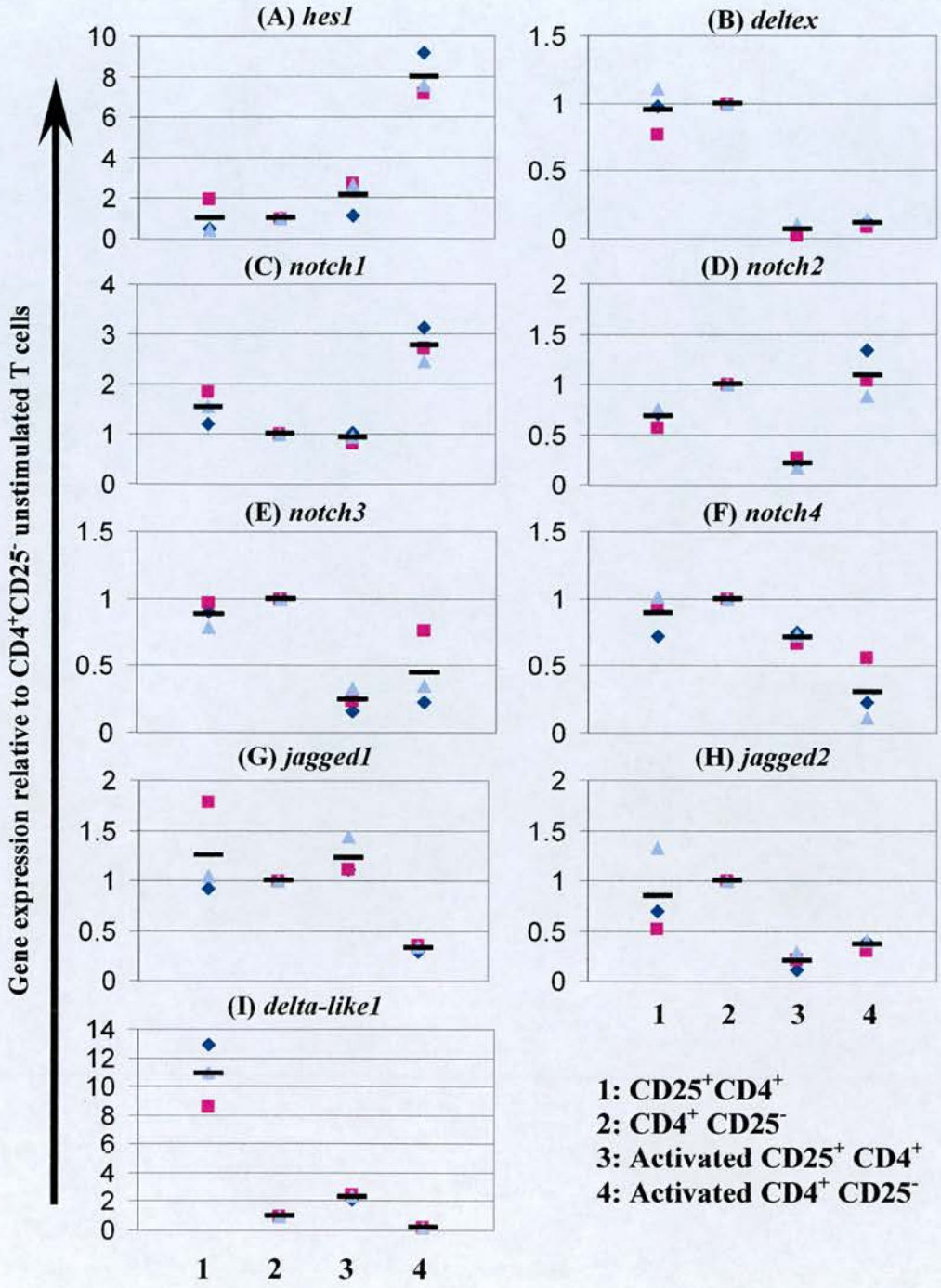


Figure 5.4: Expression of Notch pathway components by $CD25^+CD4^+$ T cells. Real-time RT-PCR was used to assess expression of Notch pathway associated genes by $CD25^+CD4^+$ T cells. RNA was extracted from $CD25^+$ and $CD25^-$ $CD4^+$ T cells, unstimulated (with APCs) or anti-CD3/APC activated. APCs were mitomycin C treated. Gene expression is presented relative to $CD4^+CD25^-$ cells. Fold changes of 2.5 or more are considered biologically relevant. Three individual experiments are each represented by as one colour of icon; the average value is depicted as a black bar.

5.4 Notch inhibition and $CD25^+CD4^+$ T cell function

Over-expression of Notch ligands on APCs is believed to induce differentiation of T reg populations. This does not address the role of Notch in mediating suppression by these cells. A possible role for Notch signalling in active regulation is hinted at by increased expression of *jagged1* and *delta-like1* by $CD25^+CD4^+$ T cells (Chapter 5.3, page 143). The potential role for Delta-like1 in mediating suppression is supported by an observed association of tolerance to house dust mite antigen with increased expression of this gene. Additionally, retroviral mediated over-expression of Delta-like1 by $CD4^+$ T cells in the later system was reported to mediate linked suppression.

This chapter aimed to block Notch signalling in $CD25^+CD4^+$ T cell suppression cultures, to determine whether Notch can mediate active regulation. Co-cultures of $CD25^+CD4^+/CD4^+CD25^-$ T cells stimulated with anti-CD3-Ab/APCs (as described in chapter 5.2, page 137) were treated with the γ-secretase inhibitor MW167 (chapter 4, page 104). Cells were then assessed for inhibition of *hes1* transcription by real-time RT-PCR to determine abrogation of Notch signalling. Proliferation and secretion of IL-10 were also measured as indicators of active regulation and Notch inhibition respectfully.

In previous experiments, use of the γ-secretase inhibitor at 20μM was found to be efficient in preventing Notch signalling in unstimulated, anti-CD3- and anti-CD3/28-Ab treated $CD4^+$ T cells. However, initial experiments using mitomycin C treated

APCs and anti-CD3-Ab as an activation stimulus revealed that 20μM MW167 was insufficient for Notch inhibition in $CD4^+$ T cells. Increasing the dose of γ-secretase inhibitor (GSI) to 100μM resulted in a 5.2±0.8 (n=3) mean fold decrease in *hes1* expression by anti-CD3-Ab/APC stimulated $CD4^+CD25^-$ T cells (Figure 5.5). $CD25^+CD4^+$ T cells also stimulated in this fashion exhibited low levels of *hes1*, mean relative expression 0.09±0.06. Treatment of activated $CD25^+CD4^+$ T cells with GSI had little effect on *hes1* expression (mean relative expression 0.07±0.05), most likely due to extremely low *hes1* expression by these cells normally.

Co-culture of $CD25^+CD4^+$ with $CD4^+$ target T cells resulted in considerable elevation in *hes1* expression, with mean relative expression values of 60±2.7 and 86.8±7.7 for ratios of 1 to 2 and 1 to 1 respectfully (Figure 5.5). Addition of γ-secretase inhibitor dramatically reduced *hes1* expression in both these groups, reducing mean relative *hes1* expression to 0.3±0.2 for 1 to 2 and 0.2±0.18 for 1 to 1 ratios of $CD25^+CD4^+$ to $CD4^+CD25^-$ T cells (Figure 5.5).

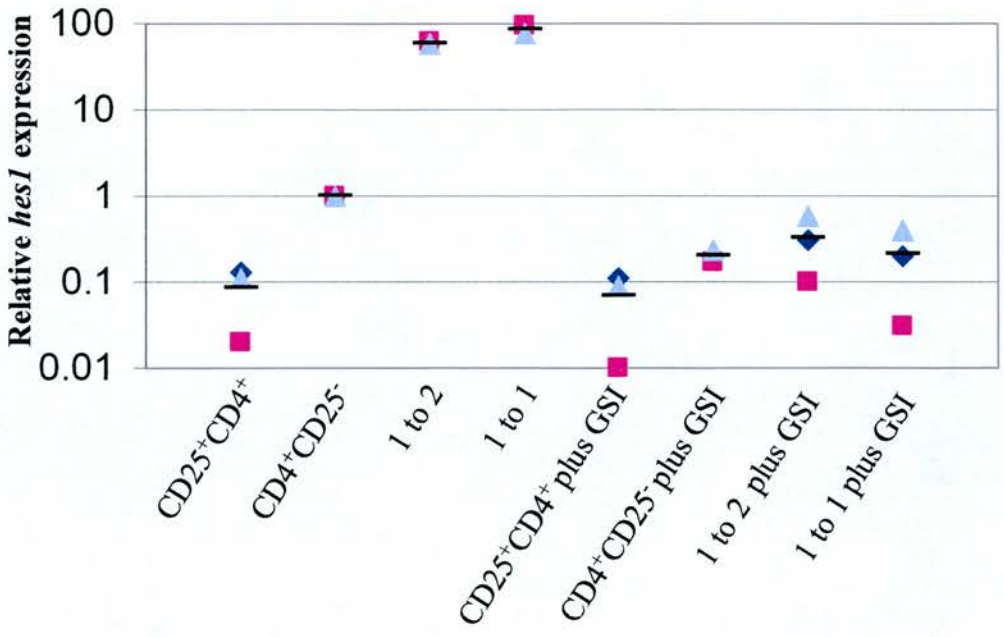


Figure 5.5: γ-secretase inhibition prevents up-regulation of hes1 expression in $CD25^+CD4^+$ T cell cultures. $CD25^+CD4^+$ T cell/$CD4^+CD25^-$ T cell co-cultures were stimulated with mitomycin C treated splenocytes and anti-CD3-Ab in the absence or presence of 100μM γ-secretase inhibitor (GSI), using DMSO as a solute control. Cells were harvested at 48 hours and total RNA extracted. Real-time RT-PCR was used to determine expression of hes1 relative to $CD4^+CD25^-$ cells. Fold changes of 2.5 or more are considered biologically relevant. Three individual experiments are each represented by as one colour of icon; the average value is depicted as a black bar.

Proliferation of $CD25^+CD4^+$, $CD4^+CD25^-$ and co-cultures in the absence of γ-secretase inhibitor (Figure 5.6A) was found to be consistent with that observed previously (chapter 5.2, page 137). Addition of 100μM MW167 to cultures did not affect proliferation of $CD25^+CD4^+$ T cells, remaining hyporesponsive in comparison to $CD4^+CD25^-$ T cells (Figure 5.6A). Proliferation of $CD4^+CD25^-$ cells was also unaffected by Notch inhibition (chapter 4.4, page). Addition of MW167 to co-cultures neither significantly inhibited nor augmented proliferation (Figure 5.6A), confirming that Notch signalling does not mediate the suppressor function of these T regs.

Analysis of IL-10 secretion revealed reduced levels in supernatants from γ-secretase inhibitor treated groups ($p<0.01$ for all groups, n=3) (Figure 5.6B), again consistent with previous findings detailed elsewhere in this thesis (chapter 4.6, page 119). Interestingly, even though suppression of $CD4^+CD25^-$ proliferation on co-culture was dramatic, an equivalent decrease in IL-10 was not observed, with only slight decreases being observed in 1 to 2 and 1 to 1 ratio cultures, also previous observed (chapter 5.2, page 137). It must be noted that these groups were found to have extremely high levels of *hes1* expression (Figure 5.5).

To further confirm that MW167 treatment could not prevent suppressor function by $CD25^+CD4^+$ T Cells, active regulation co-cultures were prepared as above but using anti-CD3-Ab/APC as an activation stimulus and with CFSE labelled $CD4^+CD25^-$ target cells in the absence or presence of the γ-secretase inhibitor (Figure 5.7 and 5.8 respectfully). Cultures were harvested after 48 hours and CFSE staining analysed by flow cytometry. Addition of γ-secretase inhibitor had no bearing on active regulation or proliferation of $CD4^+$ labelled cells alone (Figure 5.7 and 5.8). Target cells were seen to go through five rounds of division in all cases, plus or minus MW167 and even when cultured at a 1 to 1 ratio of $CD25^+CD4^+$ T regs to $CD4^+CD25^-$ cells (Figure 5.7 and 5.8). Addition of $CD25^+CD4^+$ T reg cells to labelled target cells did reduce the number of cells going through successive rounds of division, reflected in the decrease in events in gates R3, R4 and R5, combined with increases in R6, R7 and R8. Where active regulation was evident, cell appeared to have smaller forward scatter profiles (Figure 5.7C, D and 5.8C, D), the meaning of this remains to be determined.

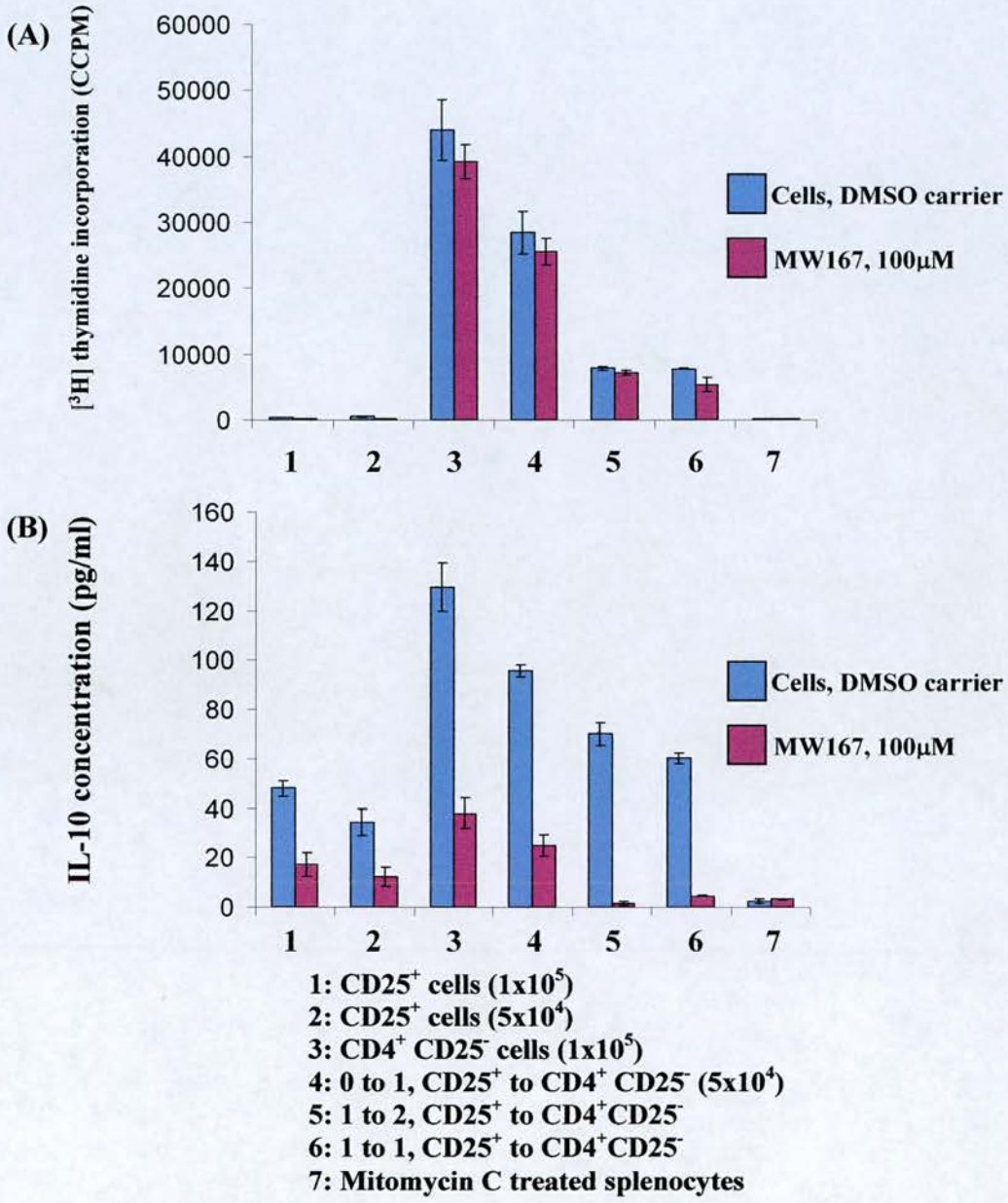


Figure 5.6: Regulation by $CD25^+CD4^+$ T cells does not depend on Notch signalling or the presence of IL-10. $CD25^+CD4^+$ T cell regulation cultures were prepared as previously described. Cells were cultured in the presence or absence of 100µM MW167 for the duration of the experiment. Overall proliferation was measured by [^{3}H] thymidine incorporation (A). IL-10 secretion was measured by ELISA (B). Cells were harvested at 72 hours. A representative experiment of three is shown. Error bars represent the standard deviation in the mean of four experimental readings.

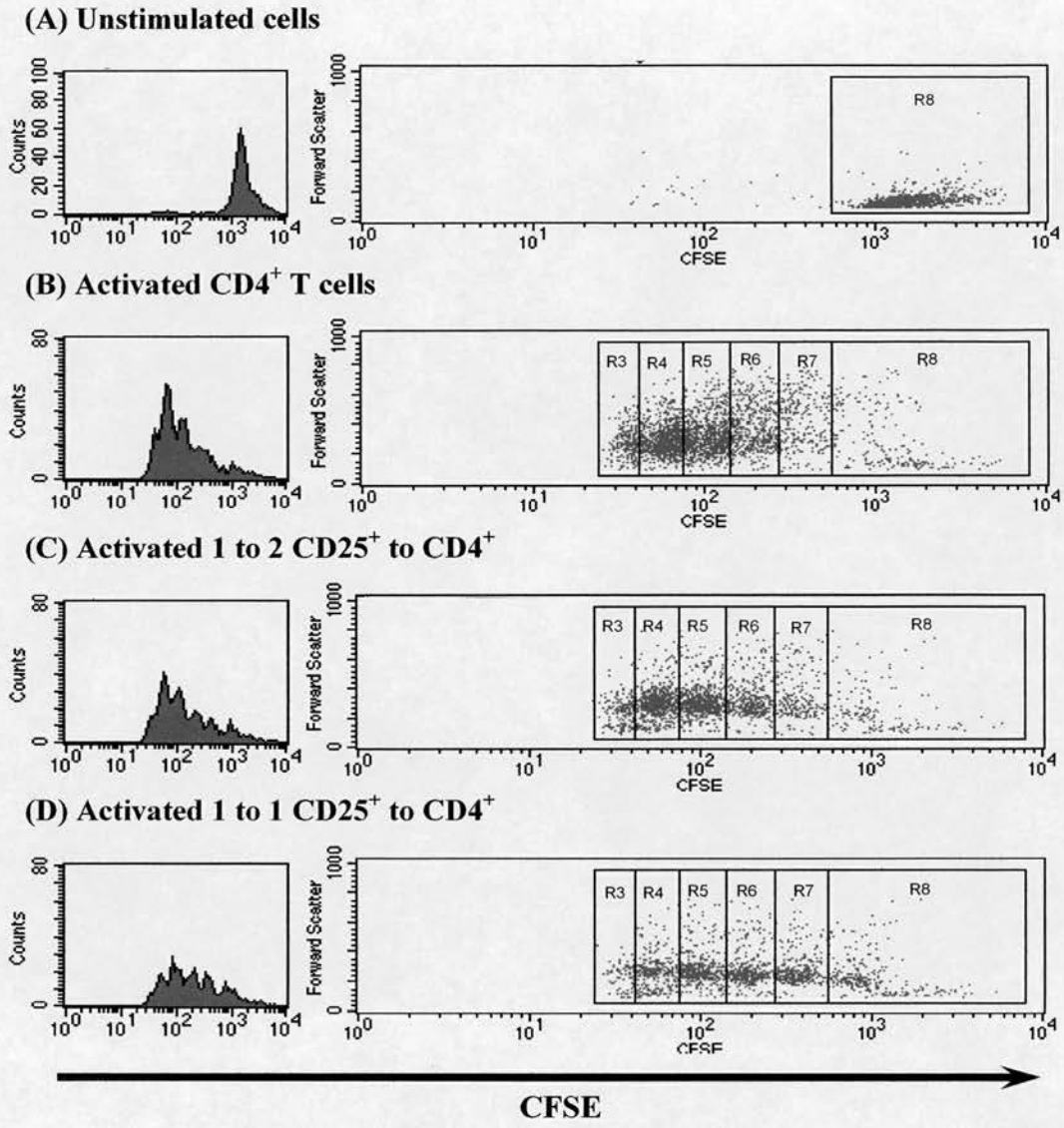


Table 5.1: Percentage of events falling in gates R3 to R8 for Figure 5.7A-D

	R3	R4	R5	R6	R7	R8
(A) Unstimulated	**0.16**	**0.71**	**0.26**	**0.13**	**0.29**	**98.51**
(B) Activated $CD4^+CD25^-$	**8.05**	**33.9**	**28.6**	**16.45**	**8.52**	**5.12**
(C) Activated 1 to 2	**7.97**	**31.34**	**27.05**	**16.66**	**8.95**	**7.54**
(D) Activated 1 to 1	**4.53**	**17.11**	**24.45**	**22.49**	**17.54**	**14.85**

Figure 5.7: Suppression of $CD4^+CD25^-$ T cell division by $CD25^+$ T regs. $CD25^+CD4^+$ T cell regulation cultures were prepared as previously described (Chapter 2.2.4, page 70). CFSE labelled $CD4^+CD25^-$ T cells were used as target cells. Cells were stimulated with anti-CD3-Ab coated, mitomycin C treated splenocytes. Cells were harvested at 48 hours and CFSE labelling examined by flow cytometry.

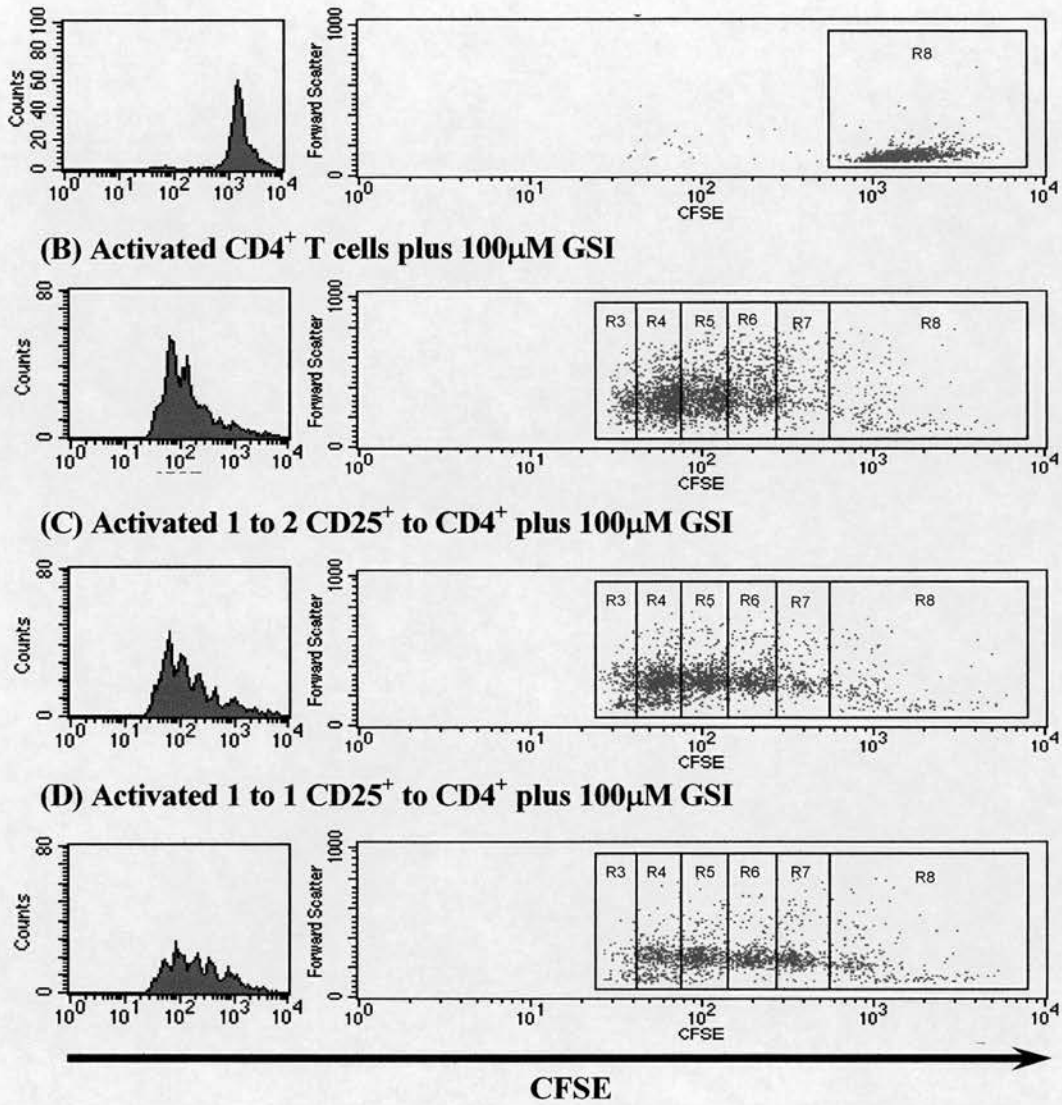


Table 5.2: Percentage of events falling in gates R3 to R8 for Figure 5.8A-D

	R3	R4	R5	R6	R7	R8
(A) Unstimulated plus GSI	0.16	0.71	0.26	0.13	0.29	98.51
(B)Activated$CD4^+CD25^-$ plus GSI	7.02	32.97	28.6	17.27	8.41	6.79
(C) Activated 1 to 2 plus GSI	8.16	31.3	27.05	17.26	8.18	8.69
(D) Activated 1 to 1 plus GSI	4.32	20.6	24.45	19.77	17.05	11.52

Figure 5.8: Suppression of $CD4^+CD25^-$ T cell division by $CD25^+$ T regs is Notch independent. $CD25^+CD4^+$ T cell regulation cultures were prepared as described in Figure 5.6. Cells were stimulated with anti-CD3-Ab coated, mitomycin C treated splenocytes in the presence of 100μM γ-secretase inhibitor. Cells were harvested at 48 hours and CFSE labelling examined by flow cytometry.

5.5 Tr1 cell generation

OVA specific T regulatory 1 (Tr1) cells were generated from the DO11.10 TCR transgenic mouse. Generation of this population was achieved by repeated stimulation in the presence of IL-10, as outlined by Herve Groux et al in 1997 (50). These cells are characterised by their lack of proliferation, high IL-10 secretion and ability to suppress proliferation of $CD4^+$ T cells in response to antigen.

Whole DO11.10 spleen was homogenised and pulsed with OVAp in the presence of IL-10 plus IL-2 or IL-2 alone to generate a Tr1 and Th cell line respectfully. Cells were then restimulated one week after previous antigen challenge. This was repeated three times before testing cell phenotype. Some of the major obstacles encountered in this section of work derived from the inability of Tr1 cells to proliferate. Addition of IL-2 with IL-10 allowed minimal Tr1 cell expansion. The use of supernatant from ConA stimulated spleen cells to expand cells was successful but prevented generation of the Tr1 phenotype. As such the number of Tr1 cells was always a limiting factor. Following successful induction of a Tr1 phenotype, lines never survived beyond another three or four subsequent expansions, the most likely explanation being exhaustion. Fortunately, sufficient Tr1 cells were derived from this system to show regulatory capabilities and assess resting cells for Notch pathway component expression.

Th cells were used as target cells for Tr1 regulation. Cells were stimulated with 0.3μM pulsed BALB/c splenocytes, as Tr1, Th or co-cultures of both cell lines. $1x10^5$ Tr1 or Th cells were cultured with splenocytes as controls for maximal cell proliferation, enabling exclusion of nutrient depletion as a determinant of reduced T cell expansion. A set number of $5x10^5$ Th cells were used as targets for suppression by Tr1 cells. Several attempts were made in generating Tr1 cells before success was achieved. The functional Tr1 line derived was designated AT2. In three out of three experiments using AT2, Tr1 cell proliferation was significantly lower than equivalent Th proliferation ($p<0.001$) (Figure 5.9). Addition of Tr1 cells to Th cell cultures at a ratio of one to 2 reduced Th proliferation, being reduced further when a 1 to 1 ratio was used ($p<0.01$) (Figure 5.9). Previous trials using mitomycin C treated splenocytes failed to demonstrate regulation, pertaining to the requirement for viable APCs to mediate suppression.

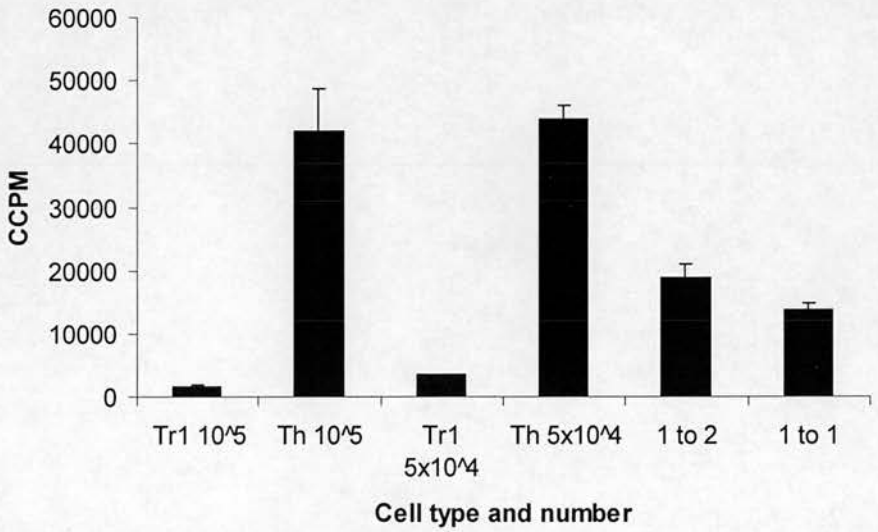


Figure 5.9: Tr1 cells inhibit Th cell line proliferation. Tr1 cells, Th cells or Tr1/Th co-cultures were activated with OVAp pulsed BALB/c splenocytes. Splenocytes were pulsed with 0.3μM OVAp for 1 hour then washed to remove residual peptide. Pulsed splenocytes were plated in 96 well plates at $1x10^6$ cells per well. Tr1 and Th cells were plated as donated by the x-axis, "1 to 2" and "1 to 1" represent ratios of "Tr1 to Th". 1 to 2: $5x10^4$ Tr1 to $1x10^5$ Th and 1 to 1: $1x10^5$ Tr1 to $1x10^5$ Th. Proliferation was measured at 72 hours by [^{3}H] thymidine incorporation. Data shown is representative of three independent experiments.

Tr1 cell activation is associated with high levels of IL-10 secretion, also believed to down-regulate APC function in the system and thus attenuate T cell proliferation. Supernatants from AT2 Tr1/Th co-cultures were collected and the presence of IL-10 assessed by ELISA. Tr1 cells were found to secrete significantly higher levels of IL-10 than Th cells ($p<0.01$ n=3) (Figure 5.10). Titration of Tr1 cells into Th cell cultures up to a ratio of 1 to 1, significantly increased IL-10 levels compared to Th cells alone ($p<0.01$ n=3) (Figure 5.10).

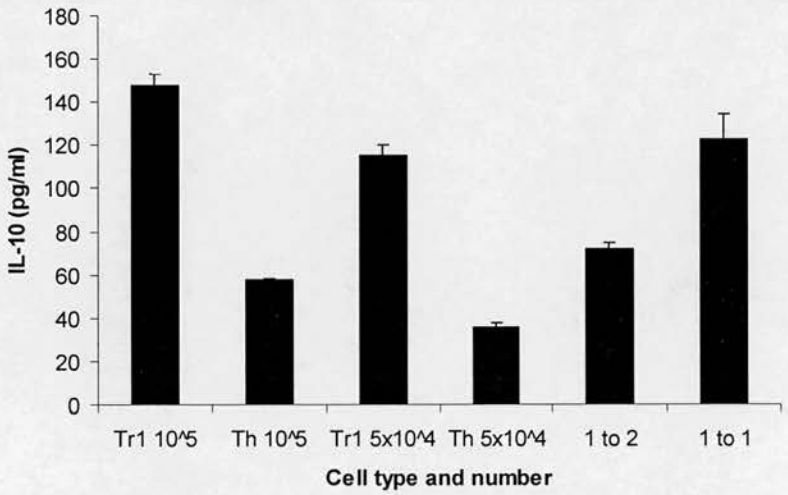


Figure 5.10: IL-10 is secreted by Tr1 cells and is associated with regulation. Tr1 cells, Th cells or Tr1/Th co-cultures were activated with OVAp pulsed BALB/c splenocytes. Splenocytes were pulsed with 0.3μM OVAp for 1 hour then washed to remove residual peptide. Pulsed splenocytes were plated in 96 well plates at $1x10^6$ cells per well. Tr1 and Th cells were plated as donated by the x-axis, "1 to 2" and "1 to 1" represent ratios of "Tr1 to Th". 1 to 2: $5x10^4$ Tr1 to $1x10^5$ Th and 1 to 1: $1x10^5$ Tr1 to $1x10^5$ Th. IL-10 was measured by ELISA from supernatants taken at 72 hours. Data shown is representative of three independent experiments.

A preliminary experiment investigating cytokine secretion in Tr1/Th co-culture systems revealed AT2 Tr1 cells secreted low level TNFα, while Th cells secreted high concentrations of this cytokine (Figure 5.11A). AT2 Tr1 cells secreted higher levels of IFNγ than Th cells (Figure 5.11B). Secretion of IL-4 or IL-5 by AT2 Tr1

cells was not detected (Figure 5.11C, D). Secretion of these Th2 cytokines by Th cells was in contrast very high (Figure 5.11C, D).

TNFα secretion in co-culture groups was found to decrease with proliferation, falling from 2214pg/ml to 267pg/ml (Figure 5.11A). IFNγ secretion in 1 to 2 cultures was found to slightly increase but was not detected when cells were cultured 1 to 1. IL-4 levels were still high in 1 to 2 ratio co-cultures, but undetectable at a cell ratio of 1 to 1 (Figure 5.11C). The same trend seen for IL-4 secretion was observed for IL-5 (Figure 5.11D).

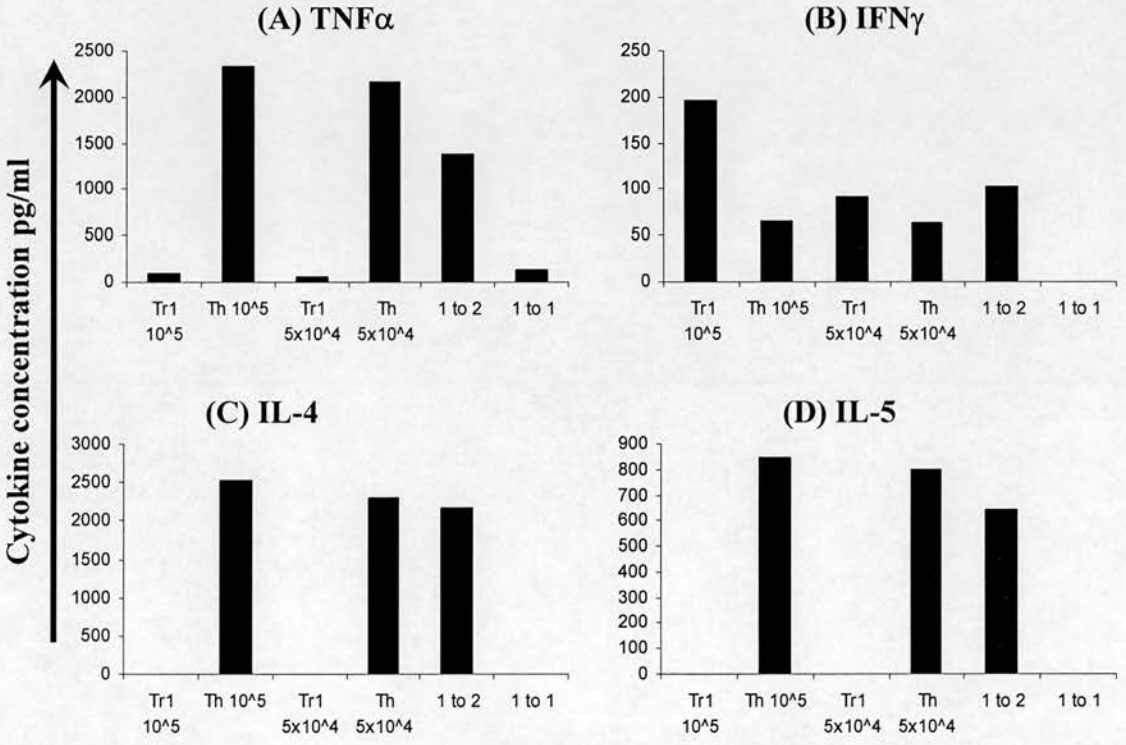


Figure 5.11: Cytokine secretion by Tr1 cells and in inhibition assays. Cytometric bead analysis was used to assess secretion of TNFα, IFNγ, IL-4 and IL-5 by Tr1, Th cells and co-cultures (as shown along the x-axis). Only one CBA was carried out due to limiting volumes of supernatant.

5.6 Notch genes and Tr1 cells

Tr1 and Th cells were rested for one week after completion of the generation protocol (Chapter 2.2, page 66). Total RNA was then extracted for a comparative analysis of Notch pathway gene expression between Tr1 and Th lines by real-time RT-PCR. Resting Tr1 cell expression of Notch receptors, ligands and downstream signalling targets was determined relative to expression by resting Th cells. Relative fold changes of 2 or more were considered biologically relevant.

Tr1 cells expressed considerably higher levels of *hes1* than Th cells, having a mean relative expression value of 11.1±1.6 (Figure 5.12A). No biologically relevant difference in *deltex* or *notch1*, *2*, *3* or *4* expression was observed (Figure 5.12B, C, D, E, F respectfully). Increased expression of *jagged1* was evident in Tr1 cells, with a mean relative expression of 2.75±0.3 (Figure 5.12G). No change in *jagged2* expression was apparent (Figure 5.12H). High levels of *delta-like1* expression was detected in Tr1 cDNAs, with a relative expression of 5.76±0.64 (Figure 5.12I).

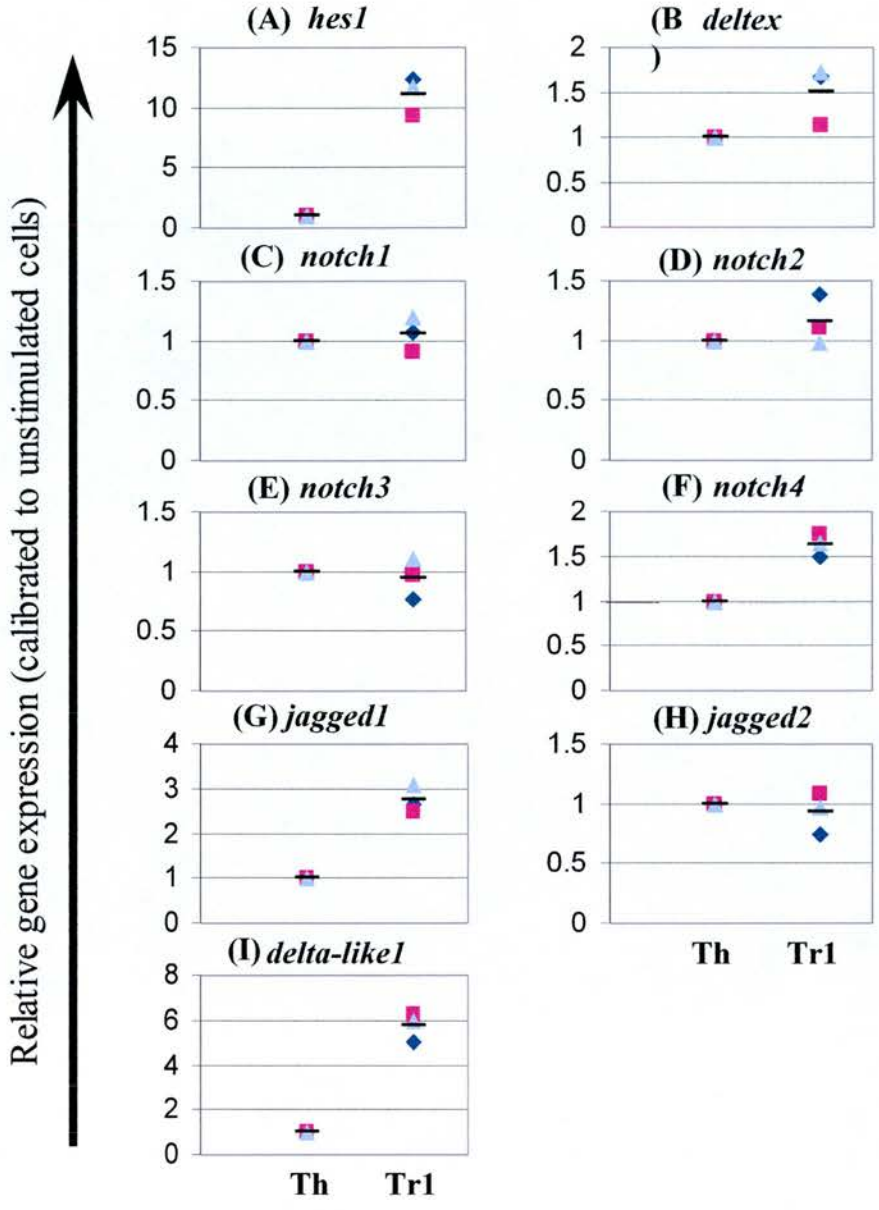


Figure 5.12: Relative expression of components of the Notch signalling pathway by Tr1 cells. Real-time RT-PCR was used to assess expression of Notch pathway associated genes by Tr1 cells. RNA was extracted from Tr1 and Th cell lines one week post stimulation. Gene expression is presented relative to the Th cell line. Three individual experiments are each represented as one colour of icon; the average value is depicted as a black bar. Fold changes of 2 or more are considered biologically relevant.

5.7 Discussion

Tolerance to a myriad of antigens has been attributed to the activity of regulatory $CD4^+$ T cells (46, 165, 24). IL-10 secretion has been associated with active regulation, although not always responsible for mediating tolerance (167). Given the impaired secretion of this cytokine by $CD4^+$ T cells in the absence of Notch, this chapter focused on how Notch signalling may be related to T reg cell function. Two populations of T reg cells were used in this study and both were found to differentially express components of the Notch signalling pathway when compared to non-regulatory counterparts. $CD25^+CD4^+$ cells expressed high levels of *delta-like1*, also observed for in vitro generated Tr1 cells. Tr1 cells expressed high levels of *hes1* and to some extent *jagged1*. Activation of $CD25^+CD4^+$ T cells maintained high *delta-like1* expression, but also induced higher *jagged1* expression than equivalent $CD25^-$ cells. Additionally, activation did not induce increased *hes1* expression as observed for $CD4^+CD25^-$ T cells. Notch did not mediate regulation by $CD25^+CD4^+$ T cells but was responsible for IL-10 secretion in culture.

Purified murine $CD25^+CD4^+$ T regs were found to mediate *in vitro* suppression in an APC dependent manner. This is consistent with the body of published literature on suppression by these murine T regs, with human cells able to regulate with out APCs present. In murine systems, the APC likely acts as a docking site allowing T reg and target to come into close proximity, allowing cell contact dependent inhibition. APC presence was also critical for suppression of *in vitro* generated Tr1 cells. Unlike

$CD25^+CD4^+$ T cell cultures, Tr1 cells required the presence of viable APCs. Tr1 cells are known to secrete high levels of IL-10, vital to active regulation by these cells. IL-10 is known to act directly on T cells, binding to and inhibiting signalling by CD28 (171, 172). However, its primary role in mediating T cell tolerance is in down-regulating MHC class II and co-stimulatory molecule expression by antigen presenting cells, thus preventing T cell activation (13, 173, 174, 175). Tr1 cell function has been demonstrated as altering the activation state of APCs consistent with IL-10 activity. The Tr1 cell phenotype of the cells used in this study (AT2) was confirmed not only by the ability to reduce proliferation of a Th cell line but also in their high secretion of IL-10. These cells are unlikely to be Th2 cells as neither IL-4 or IL-5 secretion was observed. AT2 cells did secrete higher levels of IFNγ than Th cells.

Real-time RT-PCR revealed that both $CD25^+CD4^+$ and Tr1 cells expressed high levels of *delta-like1*. $CD25^+CD4^+$ T cell expression of *delta-like1* remained high after activation, but was also accompanied by increased *jagged1* expression. These findings in murine $CD25^+CD4^+$ T cells are in line with data on human $CD25^+CD4^+$, also shown to express high *delta-like1* transcripts upon activation (176). Both Delta-like1 and Jagged1 have been implicated in mediating tolerance (1, 177). Increased expression of *delta-like1* transcripts were reported in airways of mice tolerised to the house dust mite antigen Der p1 (116). Retroviral transfection of murine $CD4^+$ T cells with *delta-like1* produced a population of T cells capable of mediating tolerance in the aforementioned system (116). Over-expression of Jagged1 by APCs also induced a population of regulatory T cells able to transfer tolerance to Der p1 (1). Both these

systems involve the inhibition of Th2 responses, however, over-expression of Jagged1 on APCs was also shown to suppress alloresponses through generation of a Th3 population (177). Combined with the finding of enhanced Notch ligand expression by well established populations of T regs, these ligand over-expression studies would imply that Notch signalling mediates active regulation. This promoted the use of the γ-secretase inhibitor to dissect out the role of Notch in mediating suppression by T regs.

Activation of Notch signalling in T lymphocytes is normally associated with lymphoma development (88). This effect is usually mediated by truncations of the Notch receptor itself and is independent of ligand expression, but it remains likely that there is a qualitative element to Notch signalling supplied direct by ligand type. Indeed, Notch receptor preference for ligand binding is directed by glycosylation, mediated by the fringe family of glycosyltransferases (178, 179). It seems unlikely that such a mechanism would have evolved and be so highly conserved if it had no biological relevance. It was therefore initially speculated that Delta-like1 may be mediating reduced proliferation. The finding that inhibition of Notch signalling prevented IL-10 secretion by $CD4^+$ T cells suggested that perhaps the role of Notch in T reg cell function was indirect, being mediated by IL-10. The role of Notch in mediating regulation was supported by the finding that extraordinarily high levels of *hes1* expression were detected in $CD25^+CD4^+/CD4^+CD25^-$ T cell cultures. Addition of γ–secretase inhibitor to these cultures blocked up-regulation of *hes1* transcription, but did not influence regulation. Secretion of IL-10 in this system mirrored transcription of *hes1*. This data reflects the finding that $CD25^+CD4^+$ active regulation

is not mediated by IL-10. Indeed, the primary mechanism has already been shown to be contact dependent (180, 181), but the data presented here confirms that $CD25^+CD4^+$ T cells regulation is not mediated by Notch signalling.

Blockade of Notch signalling in Tr1 cell active regulation cultures was not tested. This was due primarily to time restraints arising from the difficulty in generating functional Tr1 cells. However, it seems likely that Notch inhibition in this system would have yielded very different results from those seen in $CD25^+CD4^+$ T cells studies. This speculation arises from the inherent dependency of Tr1 cell generation and function on the secretion of IL-10 (182). Examination of these cells strengthens the link between Notch and IL-10, with Tr1 cells having high levels of *hes1* transcription and IL-10 secretion. High expression of *delta-like1* by these T regs may relate to a homeostatic function, maintaining the high IL-10 secretion phenotype. Treatment of activated $CD4^+$ T cells with a Delta-like1-Fc fusion protein is reported to induce cells with Tr1 like cytokine profiles (107).

Evidently expression of the Notch ligand Delta is related to $CD4^+$ regulatory T cell phenotype. The role of this molecule in mediating active regulation is still unclear, but seems to relate to IL-10 secretion. The requirement/role of IL-10 in active regulation varies from model to model, being critical in some and irrelevant in others. What is clear is that IL-10 does have immunoregulatory properties and can be utilised in generating tolerance to self and foreign antigen. Further dissection of the

role of Notch signalling induced by Delta will prove useful in deciphering its role in tolerance.

5.8 Future work

It would be of interest to generate another line of Tr1 cells for testing in the context of γ-secretase inhibition. Given the importance of IL-10 in mediating regulation by these cells it might be expected that they would not suppress proliferation in the presence of MW167.

It has been suggested that $CD25^{+}CD4^{+}$ T cells may play a role in the generation of Tr1 cells. For this reason it would be important to purify suppressed cells from the co-cultures and ascertain whether they secrete IL-10. If this were to be the case, would such cells be generated in the presence of the γ-secretase inhibitor, i.e. does Notch signalling facilitate the generation of Tr1 cells by $CD25^{+}CD4^{+}$ T regs?

A possible link between *delta-like1*, *hes1*, IL-10 production and active regulation has been the focus of this chapter. To further establish this relationship, more direct inhibitors of the Notch signalling pathway would be of interest, possibly use of RNAi in establishing specific roles for Delta-like1 and HES1.

Indeed, the level of protein expression for Delta-like1 and Hes1 would be appropriate and complimentary to the RNA data. Attempts at flow cytometric staining for Delta-like1 have been made and are discussed in Chapter 6.3, page 174.

6 Induction of Notch signalling

6.1 Introduction/background

In a previous chapter (chapter 4.5, page 116), γ-secretase inhibitor attenuation of Notch signalling reduced TCR induced cytokine secretion in the absence of CD28 co-stimulation by $CD4^+$ T cells. Inhibition of Notch signalling also prevented IL-10 production even when co-stimulation was provided. It is accepted that γ-secretase inhibitors do target other surface receptors, such as APP (183), in addition to Notch, albeit that the relevance of these other pathways to T cell function is not yet clear. To compliment the inhibition study and allow specific effects of Notch signalling in $CD4^+$ T cells to be ascertained it was decided to stimulate Notch signal transduction. Ligation of endogenous Notch receptors using recombinant ligand over-expressed on antigen presenting cells (APCs) and over-expression of a constitutively active Notch receptor in primary $CD4^+$ T cells were the two methods adopted to further this investigation.

Use of recombinant ligands presents a method of inducing more physiological levels of Notch signalling, as expression of endogenous receptors represents a "bottle-neck" limiting the amount of signalling possible. Dendritic cells (DCs) are known to express Notch ligands (184) and would be expected to be the main physiological source of Notch stimulation for T cells *in vivo*. Dendritic cells are regarded to be the most efficient APC for activation of naïve $CD4^+$ T cells, being able to provide the

necessary co-stimulation for full T cell activation. Over-expression of the Notch ligand Delta1 on such cells would provide an efficient way of activating $CD4^+$ T cells in the presence of enhanced Notch signalling. An adenoviral construct encoding murine Delta1 was already available on undertaking this PhD and as such presented an efficient method of gene transfer. Once transfected, Delta1 expressing DCs could be used to present OVAp to $CD4^+$ T cells derived from DO11.10 TCR Tg mice.

Recombinant adenovirus gene transfer represents an effective method for transfection of non dividing cells, such as fully differentiated DCs (185). In addition, adenoviral DNA is episomal reducing the risk of insertional mutagenesis associated with retroviral gene transfer. As a result expression is only transient, but this is of little concern in the *in vitro* system in which the Delta1 adenovirus (Ad-Delta1) was to be employed as cells were not cultured past 72 hours. Adenoviral constructs used in this chapter are both E1 and E3 deleted. Removal of the E1 genes (E1A and E1B) render the virus replication deficient (113), while E3 deletion reduces immunogenicity associated with adenoviral infection (113). Entry into the target cell is mediated by viral fibre protein interaction with numerous cell surface molecules, including MHC class I and primarily through the coxsackievirus-adenovirus receptor (CAR), and some integrins (186, 187, 188, 189, 190), facilitating receptor mediated endocytosis and internalisation of the virus. Of particular note is the use of the $\alpha V\beta 5$ integrin (187, 191), expression of which is known to be up-regulated when cells are cultured with GM-CSF (192), used in generation of DC enriched populations. Generation of the viruses employed, Ad-EGFP (A kind gift of Drs R. Marr and F. Graham, McMaster University, Ontario, Canada) and Ad-Delta1, utilised

homologous recombination between viral genome encoding and promoter-driven gene of interest containing plasmids within a packaging cell line (E1 transformed HEK293 cells) (193), the resultant virions being isolated by plaque purification.

Although over-expression of ligand will only activate endogenous levels of Notch signalling, it may be difficult to attribute any effect to a single Notch receptor. Notch receptors are believed to be promiscuous in their ability to bind members of the Jagged and Delta ligand families (194, 195). Over-expression of a constitutively active Notch receptor in $CD4^+$ T cells would allow assessment of a specific Notch signal. Although this system will potentially induce artificially high levels of Notch signals, comparison to results received from ligand induced signalling will allow attribution of specific effects on $CD4^+$ T cell function to particular ligand/receptor combinations.

Over-expression of truncated Notch receptors resembling the active intracellular signalling portion is a well established method for inducing constitutively active Notch signals (114). A construct encoding a truncated constitutively active form of Notch1 was obtained from Professor Raphael Kopan (Dept. Molecular Biology & Pharmacology & the Dept. of Medicine, Washington University) (114). The insert encoding Notch1-intracellular domain (NICD1) was then sub-cloned into a vector suitable for transfection of T cells, detailed in the materials and methods section (Chapter 2.2, page 66). It must be stressed that transfection of primary $CD4^+$ T cells is very difficult, with the majority of experiments opting to use cell lines. Experiments using primary T cells have utilised retroviral gene transfer technology

(196). Since naïve T cells are not cycling cells, they need to be activated to induce proliferation allowing retroviral infection (196), negating the possibility of looking at truly naïve T cells. Additionally, transferred genes are integrated with host DNA (197), and can be associated with insertional mutagenesis. Electroporation has been used to transfect T cell lines, however, such systems are associated with poor cell viability when using primary T cells. Commercial release of nucleofection technology (Amaxa) has allowed transfection of primary human $CD4^+$ T cells (198). Unfortunately the technology has not yet been extended to include murine T cells, which this PhD has focused on. Having been presented with the opportunity to transfect primary $CD4^+$ T cells with a constitutively active Notch receptor, the cross-over to human cells was made. It is for this reason that experiments over-expressing NICD1 were performed using $CD4^+$ T cells purified from human PBMCs.

Chapter aims:

- Culture population of APCs enriched with DCs.
- Optimise viral infection protocol for DC enriched population..
- Over-express Delta1 on DCs.
- Use Ad-delta1 DCs to activate $CD4^+$ T cells, assessing proliferative and cytokine responses.
- Induce Notch1 signalling directly in human $CD4^+$ T cells using NICD1 over-expression.

6.2 Adenoviral infection of a DC enriched cell population

Bone marrow derived DCs were generated based on a previously published Inaba *et al* protocol (112). This protocol has been used extensively by others within the laboratory and was chosen as it would provide a ready supply of efficient antigen presenting cells.

Single cell suspensions were prepared from BALB/c bone marrow and seeded in 24 well plates at $3.75x10^5$ cells/ml. Cells were differentiated using hybridoma derived GM-CSF supernatant over a period of seven days (Chapter 2.2, page 66). Cells were cultured for a further 5 hours in the absence or presence of 100ng/ml LPS to induce maturation before staining for flow cytometric analysis. Cells were stained for CD11c, Gr-1, MHC class II and CD80 to give an indication of DC enrichment and granulocytic contamination (Figure 6.1). In a representative experiment, the population was found to consist primarily of $CD11c^+$ cells, 79% before and 72% after LPS stimulation (Figure 6.1G, H). 22% of cells were $Gr\text{-}1^+$, before and after LPS maturation (Figure 6.1I, J). Maturation of cells by LPS was confirmed by enhanced expression of MHC class II and CD80 after stimulation. Although the percentages of positive cells changed only slightly, the mean fluorescence intensity (MFI) of MHC class II and CD80 staining rose from 637 to 914 (Figure 6.1K, L) and 245 to 396 (Figure 6.1M, N) respectively, indicating enhanced expression of these markers on a per cell basis.

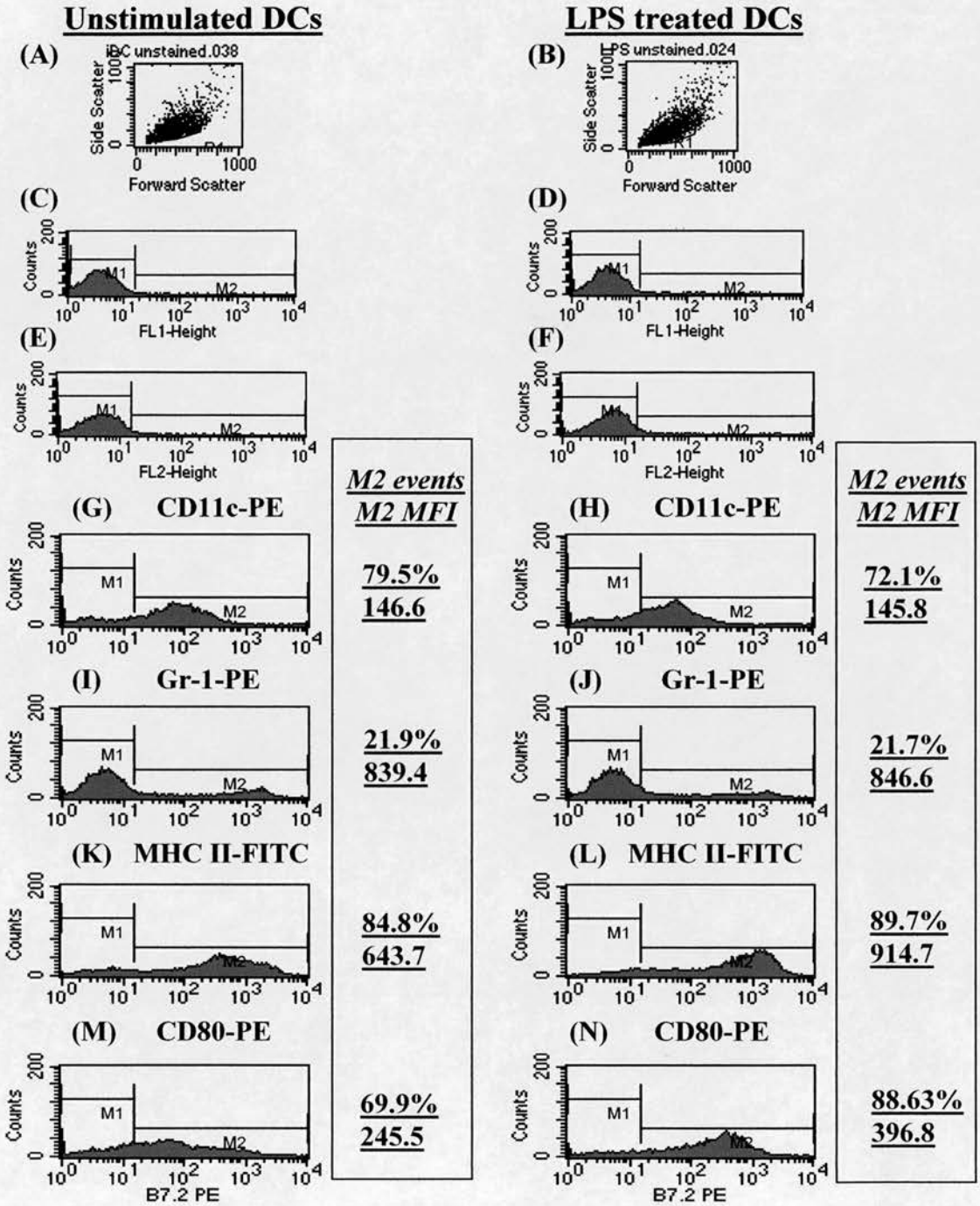


Figure 6.1: DC enriched population. DCs were generated from BALB/c bone marrow according to the Inaba *et al* method. Unstimulated and LPS treated cells (100ng/ml for 5 hours) were characterised on the basis of flow cytometric analysis. Forward and side scatter profiles for unstimulated and LPS treated cells are shown (A, B). FITC and PE isotype controls are also show (C-F). CD11c (G, H), Gr-1 (I, J), MHC class II (K, L) and CD80 (M, N) expression are depicted along with the percentage of gated events falling in M2 and mean fluorescent intensity (MFI). One experiment of two is shown.

An in house Ad-GFP construct was used to determine the optimal multiplicity of infection (MOI) for use with the DC enriched population. Briefly, DCs were plated at $3x10^6$ cells in 500µl and incubated with the adenovirus for 1 hour. Cells were then washed and re-plated at $3x10^6$ in 3ml in 6 well plates (Chapter 2.2.12, page 66). Cells were harvested after 24 hours and GFP expression assessed by flow cytometry. Ad-GFP MOIs of 25, 50, 100, 200 and 400 were used to determine to amount of virus needed to achieve a maximal transfection efficiency (Figure 6.2).

Cells were analysed for GFP fluorescence on FL-1, allowing easy quantification of infected cells (Figure 6.1A). The percentage of cells positive for GFP expression increased from 37±11% to 81±4% at MOIs of 25 and 200 respectfully (n=3) (Figure 6.1A, B). Increasing the MOI to 400 did not significantly enhance infection efficiency (Figure 6.1A, B). Since infection rates peaked at an MOI of 200, this MOI was used throughout the remainder of this chapter. The dendritic morphology of GFP positive cells was clearly visible using fluorescence microscopy (Figure 6.1C).

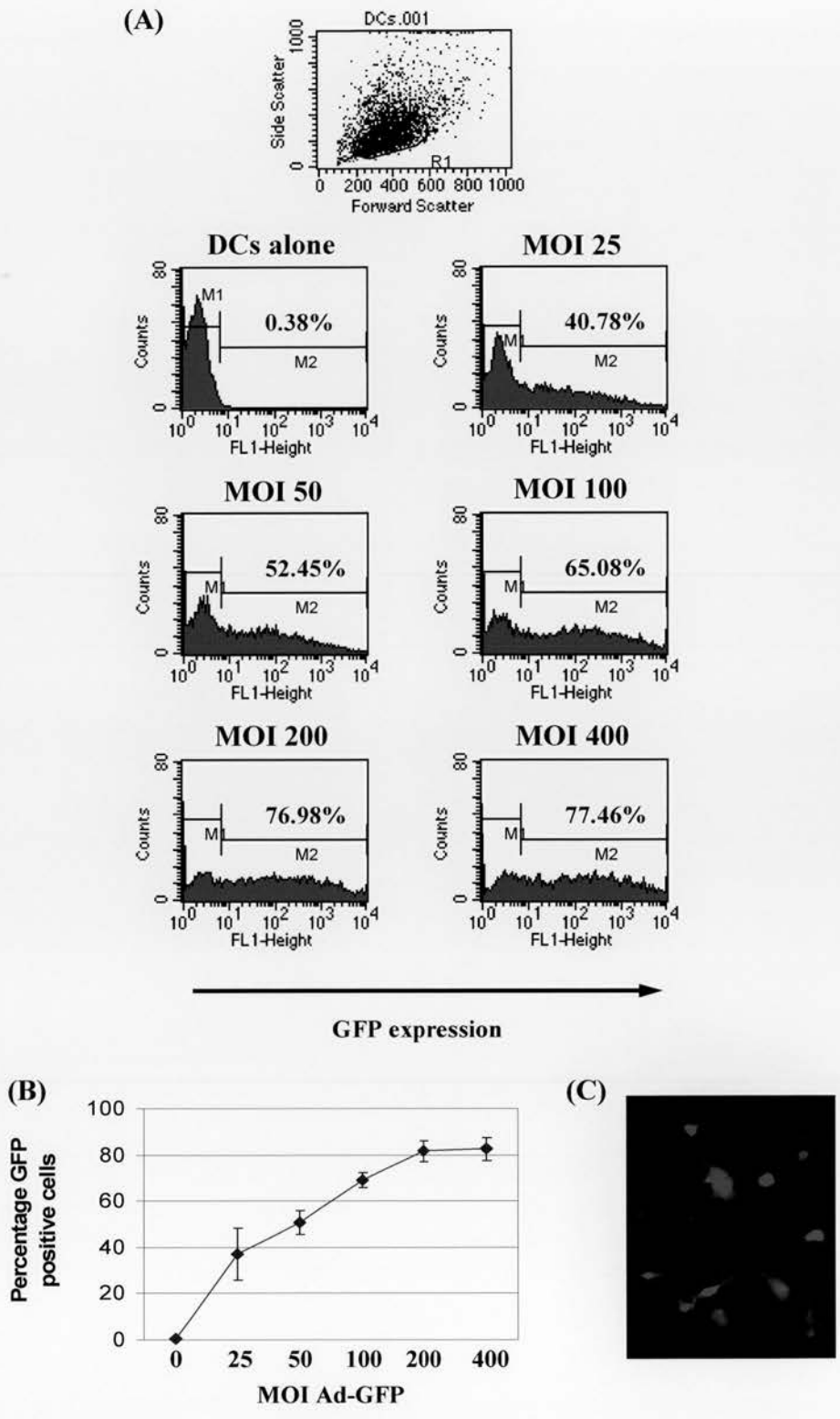


Figure 6.2: DC infection with Ad-GFP. Optimal MOI for DC infection was determined using an Ad-GFP vector. DC enriched populations with incubated with increasing MOIs of virus for 1 hour. Cells were then washed and incubated for a further 24 hours. Cells were than harvested and GFP expression determined by flow cytometry. Representative histogram plots are shown (A). The mean number of GFP positive cells from three individual titrations was also plotted (B). (C) GFP positive cells under a fluorescent microscope (X400).

6.3 $CD4^+$ T cell activation by Ad-delta1 DCs

To ascertain effects mediated by ligand induced Notch signalling on $CD4^+$ T cells, DCs infected with an adenoviral construct encoding the Notch ligand Delta1 (Ad-delta1) (Chapter 2.1, page 62) were used to stimulate naïve T cells. Detection of Delta1 protein was hampered by a lack of efficient antibodies. Much time was spent trying to develop western blot and flow cytometric protocols to establish induction of protein expression by the adenoviral construct. Western blotting was unsuccessful and although some positive results were obtained by flow cytometry (Figure 6.3), the inconsistency of the method was of concern. Real-time RT-PCR also failed to demonstrate enhanced *delta1* expression as the data was un-interpretable due to what appeared to be a non-specific viral effect interfering with the real-time reaction. Indeed, not only were genes of interest affected, interference was also observed for the 18S internal control reaction. Despite lack of detection of transgene expression, it was decided to continue with this part of the project and determine if use of the Ad-delta1 construct mediated any biological effect.

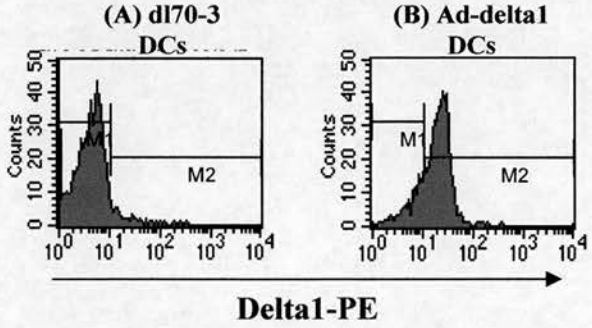


Figure 6.3: Delta1 expression. Day 7 DCs were incubated with either dl70-3 (A) or Ad-delta1 (B) for 1 hour. Cells were then washed and cultured for a further 24 hours to allow for protein expression. Cells were then stained with anti-Delta1 (T-20), followed by a biotinylated bovine anti-goat then streptavidin-PE (Detailed in Chapter 2.2.3 page 68).

Uninfected, dl70-3 (empty control vector) or Ad-delta1 treated DCs were either unstimulated or matured with LPS (believed to enhance expression of transgenes driven by CMV promoters, such as that utilised by this construct). Cells were incubated for 24 hours before mitomycin C treatment and pulsing with OVA peptide (Chapter 2.2.4, page 70). DCs were then co-cultured with purified DO11.10 TCR transgenic $CD4^+$ T cells, $1x10^4$ DCs to $5x10^4$ T cells. Proliferation was measured by $[^3H]$ thymidine, pulsed at 48 and harvested at 72 hours. Supernatants were harvested at 48 hours and cytokine secretion assessed by cytometric bead analysis and ELISA.

In three experiments, proliferation of unstimulated $CD4^+$ T cells in the presence dl70-3 or Ad-delta1 treated DCs was not found to differ significantly from that induced by untreated DCs (Figure 6.4A). Incubation of $CD4^+$ T cells with unpulsed, LPS matured DCs or LPS, dl70-3 DCs did not alter T cell division. Culture with LPS matured, Ad-delta1 infected DCs did however induce a marginal increase in non-specific $CD4^+$ T cell proliferation which was found to be significant compared to LPS DCs and LPS dl70-3 DCs, p=0.007 and 0.047 respectfully (Figure 6.4A).

In five experiments, use of specific antigen (OVAp) pulsed DCs induced a significant increase in $CD4^+$ T cell proliferation compared to unpulsed DCs (Figure 6.4A, B), p=0.021. Antigen driven proliferation was enhanced using pulsed DCs treated with either dl70-3 or Ad-delta1, p=0.007 and 0.004 respectfully. No significant difference between dl70-3 and Ad-delta1 groups were observed (Figure 6.4B). LPS matured OVAp pulsed DCs induced significantly higher levels of $CD4^+$

T cell proliferation than non LPS matured DCs, p=0.007 (Figure 6.4B). Pulsed-LPS-matured-adenovirally infected DCs were able to induce greater levels of proliferation than pulsed-LPS-uninfected DCs, p=0.05 and 0.04 for dl70-3 and Ad-delta1 pulsed-LPS-infected DCs respectfully (Figure 6.4B). No significant difference in T cell proliferation was observed between groups stimulated by pulsed-LPS-dl70-3 and pulsed- LPS-Ad-delta1 DCs.

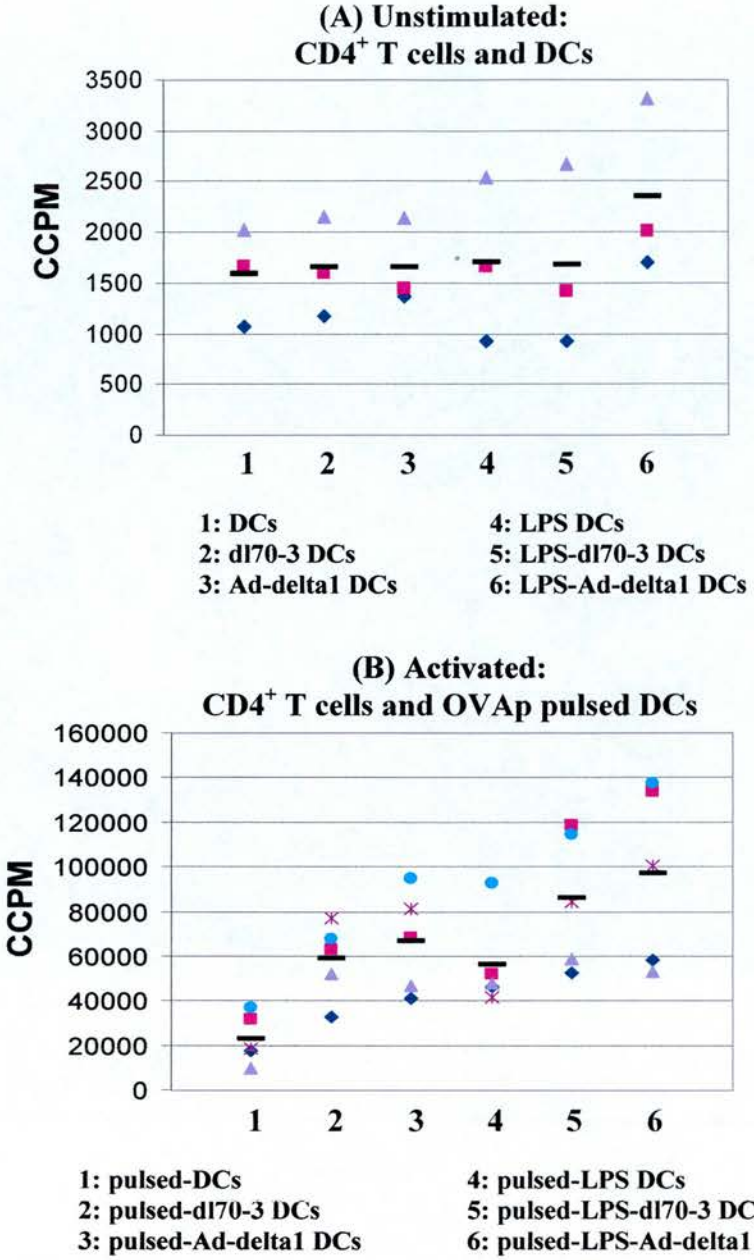


Figure 6.4: Ad-delta1 DCs and CD4$^+$ T cell activation. DC enriched populations were infected with dl70-3 or Ad-delta1, un-pulsed (A) or pulsed (B) with OVAp (0.3μM) and mitomycin C treated. DC to DO11.10 CD4$^+$ T cell ratio was at 1 to 5, proliferation was measured using [^{3}H] thymidine harvested at 72 hours. One colour of icon represents an individual experiment, the black bar depicts the mean value.

CBA and ELISA failed to detect the presence of TNFα, IFNγ, IL-4, IL-5 or IL-10 in cultures where DO11.10 $CD4^+$ T cells were not activated with specific peptide.

Analysis of supernatants from OVAp activated T cells revealed significant induction of TNFα secretion by $CD4^+$ T cells stimulated by adenovirally infected DCs when compared to uninfected DCs (dl70-3: p=0.03, Ad-delta1: p=0.02) (Figure 6.5A, B). No difference in TNFα secretion between adenoviral groups was observed.

TNFα levels were found to be significantly higher when LPS matured DCs were used rather than unstimulated DCs (p=0.01) (Figure 6.5A). No significant difference in TNFα secretion was observed between LPS-dl70-3 and LPS-Ad-delta1 adenovirally infected groups. Higher levels of TNFα were detected relative to non-infected LPS DC activated T cells (p=0.0004 and 0.007, dl70-3 and Ad-delta1 respectfully) (Figure 6.5A).

$CD4^+$ T cell IFNγ secretion was enhanced by treatment of DCs with LPS compared to non-matured DCs (p=0.004) (Figure 6.5B). Adenovirally infected DCs did not significantly enhance IFNγ secretion. No significant difference between LPS-adenoviral treated DC and LPS DC groups was observed. Neither was any significant IFNγ secretion difference between LPS-dl70-3 and LPS-Ad-delta1 DC activated T cells detected (Figure 6.5B).

IL-10 secretion by activated $CD4^+$ T cells was found to be unaffected by introduction of the control adenovirus dl70-3, but was enhanced when DCs had been treated with

Ad-delta1, p=0.046 (Figure 6.5C). A trend for elevated IL-10 secretion by activated $CD4^+$ T cells was observed in LPS matured DC groups compared to non-LPS treated DCs (Figure 6.5C). No significant difference was observed between LPS and LPS matured, adenovirally infected DC groups (Figure 6.5C).

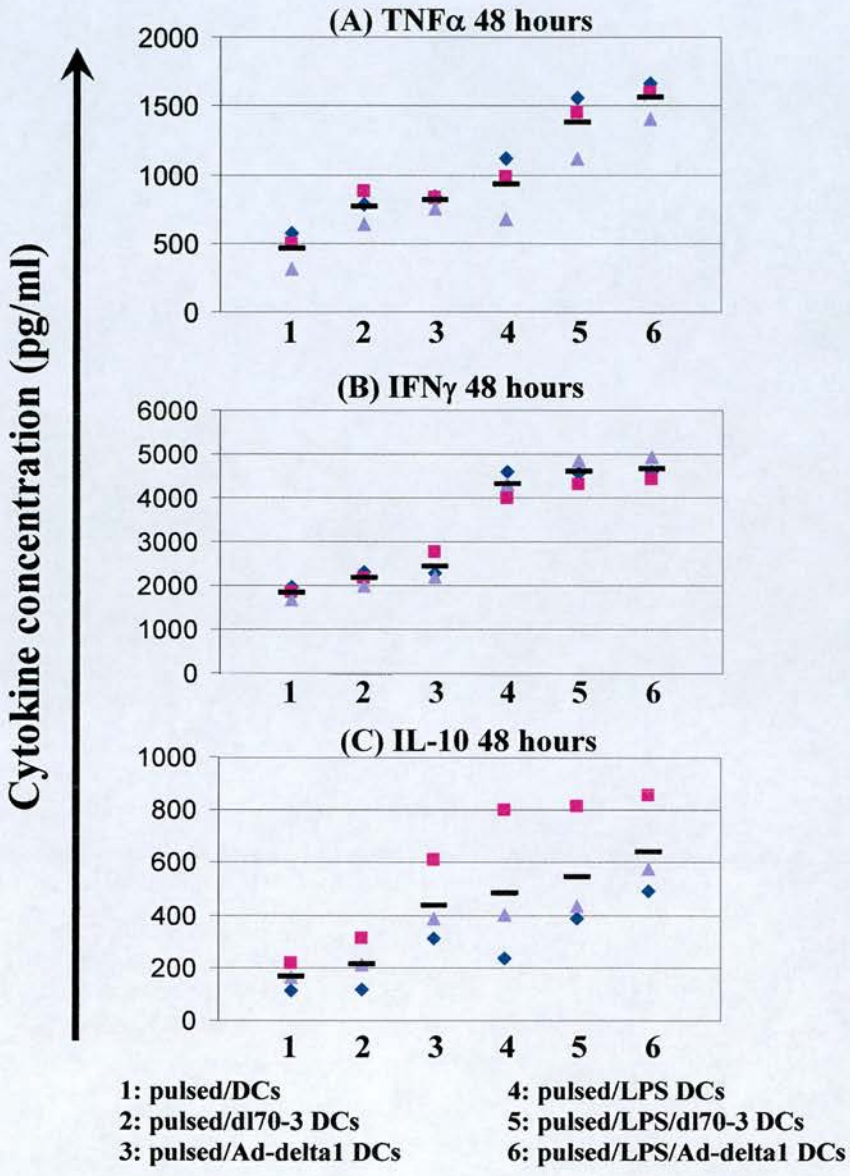


Figure 6.5: Cytokine secretion by $CD4^+$ T cells activated by adenovirus infected DCs. DC enriched populations were infected with dl70-3 or Ad-delta1, mitomycin C treated then pulsed with OVAp (0.3μM) before incubation with purified DO11.10 $CD4^+$ T cells. DC to T cell ratio was at $1x10^4$ to $5x10^4$. Supernatants were harvested after 48 hours of culture. TNFα (A) and IFNγ (B) secretion was assessed using cytometric bead analysis. IL-10 secretion (C) was determined by ELISA. One colour of icon represents an individual experiment, the black bar depicts the mean value.

Given that the primary role of DCs is to act as sentinels and alert T cells to danger associated antigens, it seemed reasonable to assume that the adenoviral constructs were perhaps activating the DCs by means of a danger signal. This signal could possibly up-regulate co-stimulatory molecules on the DCs and may help explain the observed increases in proliferation and cytokine secretion by $CD4^+$ T cells incubated with adenovirally infected DCs. Uninfected DCs, dl70-3 and Ad-delta1 treated DCs were stained for expression of CD80, CD86 and MHC class II to reveal any change in maturation state.

The uninfected DC enriched population expressed low level CD80, CD86 and MHC class II (Figure 6.6A, B and C, representative histogram plots). Incubation with control virus dl70-3 enhanced expression of all three surface markers (CD80: p= 0.013, CD86: 0.016, MHC class II: 0.006) (n=4) (Figure 6.6D, E, F). Incubation with Ad-delta1 further enhanced expression of CD80, CD86 and MHC class II relative to dl70-3 treatment (CD80: p=0.007, CD86: 0.036, MHC class II: 0.01) (n=4) (Figure 6.6G, H, I).

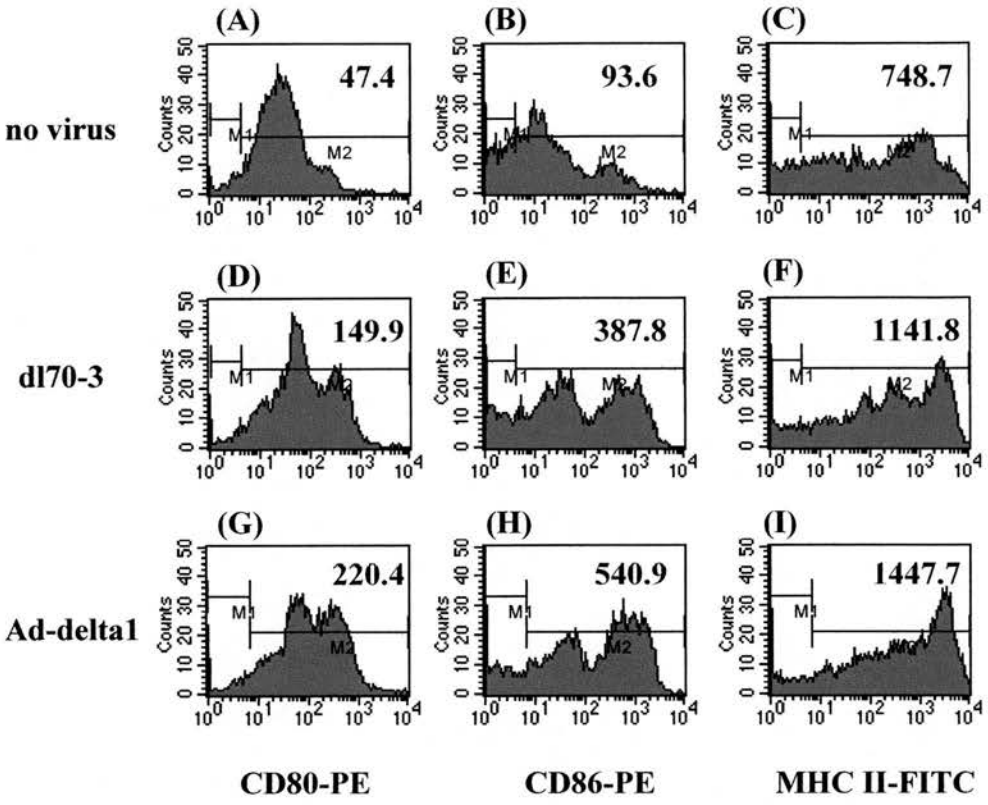


Figure 6.6: Maturation of DC enriched cell population by adenovirus. Bone marrow derived DCs were incubated without virus (A-C), control virus dl70-3 (D-F) or Ad-delta1 (G-I) for 1 hour, washed and cultured for a further 24 hours before analysis of CD80, CD86 and MHC class II expression by flow cytometry. The mean fluorescent intensity for region M2 is shown in each panel. One representative experiment of four is shown.

6.4 Over-expression of NICD1 in human $CD4^+$ T cells

Induction of Notch signalling using over-expression of ligand appeared inconclusive and as such it was decided to try and induce a specific Notch signal by transfection of $CD4^+$ T cells with a constitutively active Notch receptor. In a "Letters to nature paper" by Schroeter *et al* (114), the requirement for nuclear translocation of the intracellular domain of Notch1 in mediating signal transduction was demonstrated using truncated receptors. One of these constructs was kindly gifted by Professor Raphael Kopan (N-ICv1744) (114), encoding a myc tagged Notch1 intracellular domain. The inserted sequence was sub-cloned into a commercially available pEGFP vector to generate the plasmid pNICD1 (Figure 6.7). The insert was cloned keeping the stop codon intact to prevent generation of an EGFP fusion protein that may have affected the function of NICD1. Sub-cloning allowed use of pEGFP as a transfection control and to determine transfection efficiency.

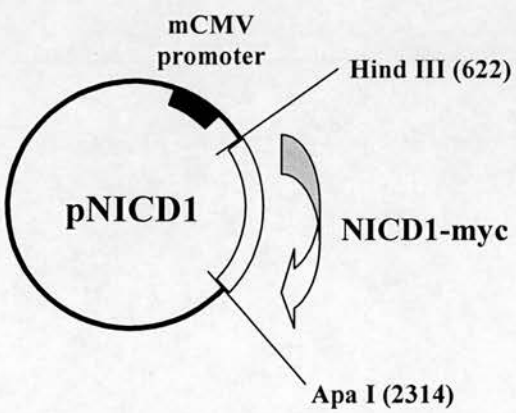


Figure 6.7: pNICD1. A myc tagged NICD1 sequence was sub-cloned into the commercially available pEGFP-N1 vector. The myc tag was followed by a stop codon preventing transcription of an EGFP fusion protein. The pEGFP-N1 vector was used as a control vector allowing estimation of transfection efficiency. Details of the sub cloning are detailed in Materials and Methods (Chapter 2.2.13, page 81).

Amaxa nucleofection technology was used to transfect human $CD4^+$ T cells (Amaxa transfection of mouse cells is currently associated with high cell mortality). Human $CD4^+$ T cells were purified by MACs isolation. Transfection of cells with 1μg pEGFP gave transfection efficiencies of 54±6% (n=3) whether cells were anti-CD3- or anti-CD3/28-Ab stimulated. Efficiency was lower, 37±4% (n=3) with unstimulated cells. Use of larger amounts of DNA was associated with a decrease in cell viability (based on forward/side scatter plots and a reduced ability of cells to respond to anti-CD3/28-Ab stimulation). Little information regarding improving transfection was available from Amaxa regarding the content of the supplied reagents so no attempt could be made to further improve efficiency.

Purified $CD4^+$ human T cells were transfected according to manufacturer's instructions with no DNA (mock), pEGFP or pNICD1. Cells were then cultured for 48 hours, unstimulated, anti-CD3- or anti-CD3/28-Ab treated. Proliferation was measured by $[^3H]$ thymidine incorporation and supernatants were assayed for cytokine secretion.

Low level proliferation by unstimulated mock transfected $CD4^+$ T cells was unaffected by transfection with pEGFP or pNICD1 (Figure 6.8). Mock transfected cells proliferated in response anti-CD3-Ab, a response enhanced slightly by addition of anti-CD/28-Ab (Figure 6.8). pEGFP transfected cells proliferated less than mock transfectants to both these stimuli (Figure 6.8), most likely due to EGFP toxic effects. Transfection with pNICD1 further reduced proliferation in response to anti-CD3- and anti-CD3/28-Ab stimulation (Figure 6.8).

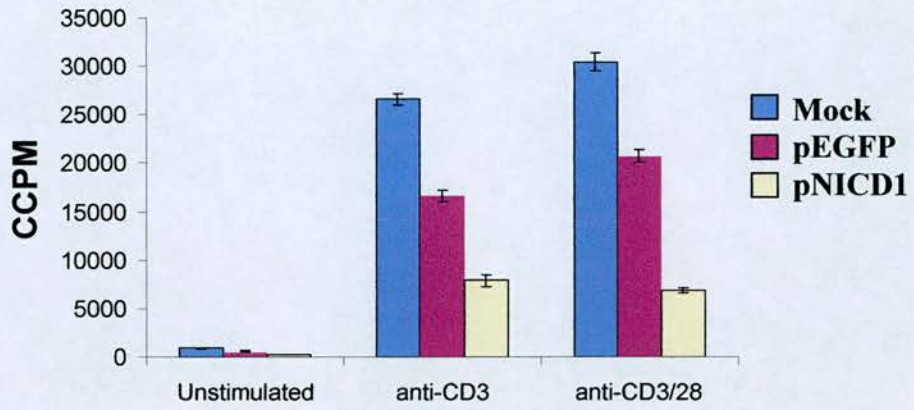


Figure 6.8: pNICD1 reduces proliferation of human CD4⁺ T cells. $CD4^+$ T cells were isolated by MACs negative selection from human PBMCs. Cells were then transfected using the Amaxa system, either as a mock transfer or in the presence of pEGFP or pNICD1. Proliferation was measured at 48 hours of unstimulated, anti-CD3 or anti-CD3/28 treated cells. One experiment is shown, error bars represent the standard deviation of four replicates.

Cytokine secretion by transfected $CD4^+$ T cells was assessed by cytometric bead analysis. Enhanced IFNγ secretion was observed by both anti-CD3-Ab stimulated pNICD1 transfectants relative to mock and pEGFP groups (Figure 6.9A). Stimulation with anti-CD3/28-Ab resulted in a similar trend, however the difference between control groups and pNICD1 was less pronounced (Figure 6.9A). The same trend was evident when TNFα secretion was assessed (Figure 6.9B). pNICD1 enhanced TNFα secretion upon anti-CD3-Ab stimulation, but with a less distinct difference detected when anti-CD3/28-Ab was used (Figure 6.9B).

pNICD1 transfection also enhanced IL-10 secretion induced by anti-CD3-stimulation (Figure 6.9C). However, a marked increase was still evident when pNICD1 cells were treated with anti-CD3/28-Ab (Figure6.9C), unlike IFNγ and TNFα (Figure 6.9A, B). This same trend was also observed for IL-4, the difference between anti-

CD3/28-Ab pNICD1 and control groups being particularly pronounced (Figure 6.9D). IL-5 secretion was increased upon stimulation in the presence of pNICD1 in comparison to mock and pEGFP transfectants. Although a trend for enhanced cytokine secretion was observed when pNICD1 cells were activated, a difference may be apparent when comparing anti-CD3- and anti-CD3/28-Ab stimulation. It might be speculated that pNICD1 transfection enhances IFNγ and TNFα upon anti-CD3-Ab treatment (Figure 6.9A, B), but when anti-CD3/28-Ab is used a more profound difference is observed in IL-4 and IL-5 production (Figure 6.9D, E). Enhanced IL-2 secretion was also observed by pNICD1 transfectants (Figure 6.9F). Unlike the other cytokines assayed, IL-2 secretion was enhanced in pNICD1 unstimulated cells as well as anti-CD3- and anti-CD3/28 stimulated transfectants relative to both mock and pEGFP groups (Figure 6.9F).

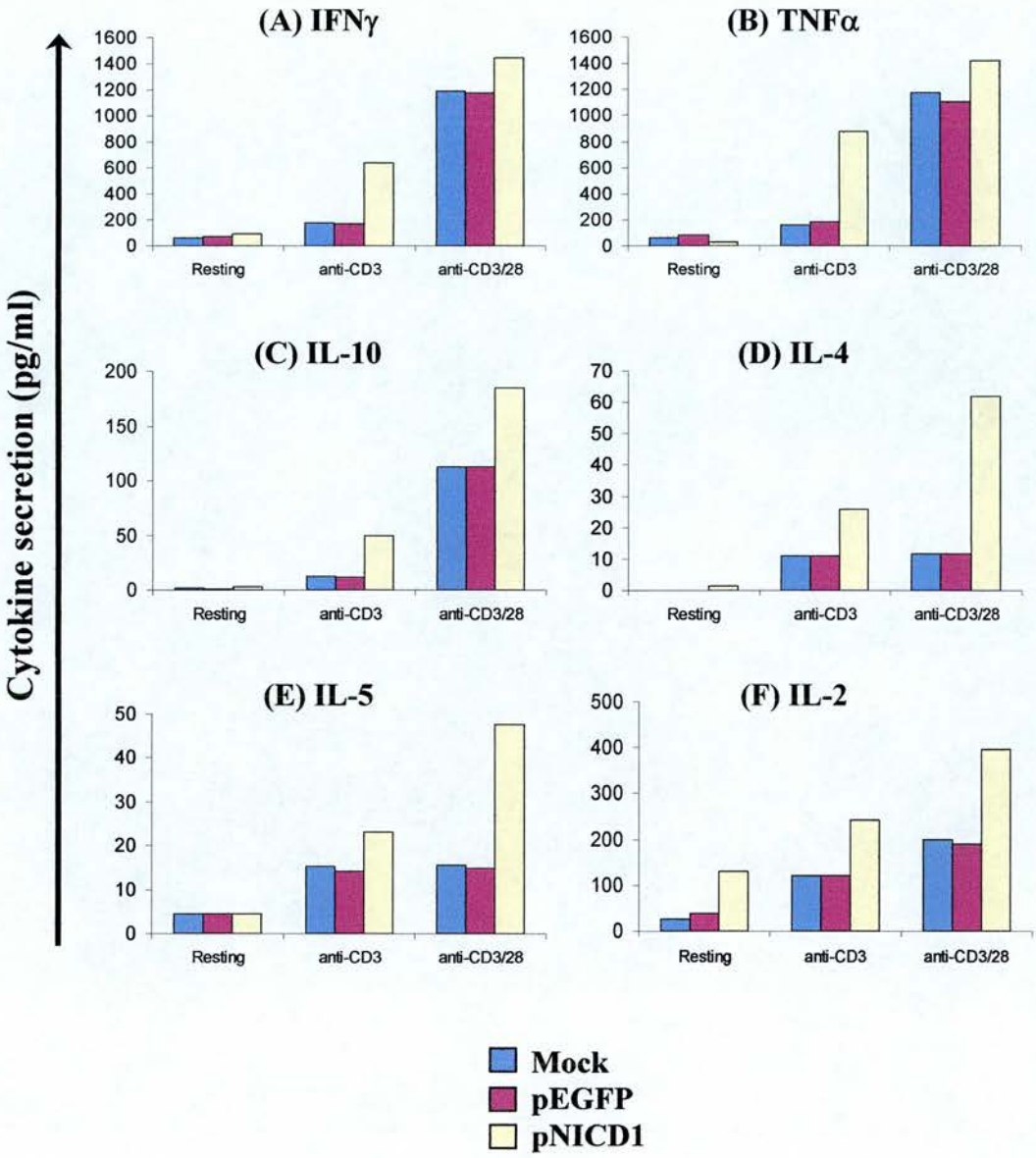


Figure 6.9: pNICD1 transfection enhances cytokine secretion by activated human CD4$^+$ T cells. CD4$^+$ T cells were isolated by MACs negative selection from human PBMCs. Cells were then transfected using the Amaxa system, either as a mock transfer or in the presence of pEGFP or pNICD1. Cytokines were harvested at 48 hours from unstimulated, anti-CD3- and anti-CD3/28-Ab treated cells. Secretion of IFNγ, TNFα, IL-10, IL-4, IL-5 and IL-10 was assessed using cytometric bead analysis. Data from an individual experiment is shown.

Real-time RT-PCR did reveal up-regulation of relative *hes1* expression in pNICD1 transfectants relative to mock cells (Figure 6.10). *hes1* expression was up 4.7 fold relative to mock unstimulated cells, and 6 fold relative to EGFP transfected cells. Anti-CD3 stimulation increased *hes1* expression relative to unstimulated cells (as in murine $CD4^+$ T cells, Chapter 3.3, page 89) in all groups. However, pNICD1 transfection induced a relative expression of 26.3 compared to 6.1 and 4.9 of mock and pEGFP anti-CD3-Ab stimulated cells respectfully (Figure 6.10). The same pattern was observed for anti-CD3/28-Ab treatment, pNICD1 transfection enhancing relative *hes1* expression from 6.67 and 5.7, mock and pEGFP respectfully, to 28.2 (Figure 6.10). Enhancement of *hes1* expression by pNICD1 implies that the construct is producing a functional protein, augmenting Notch signal transduction.

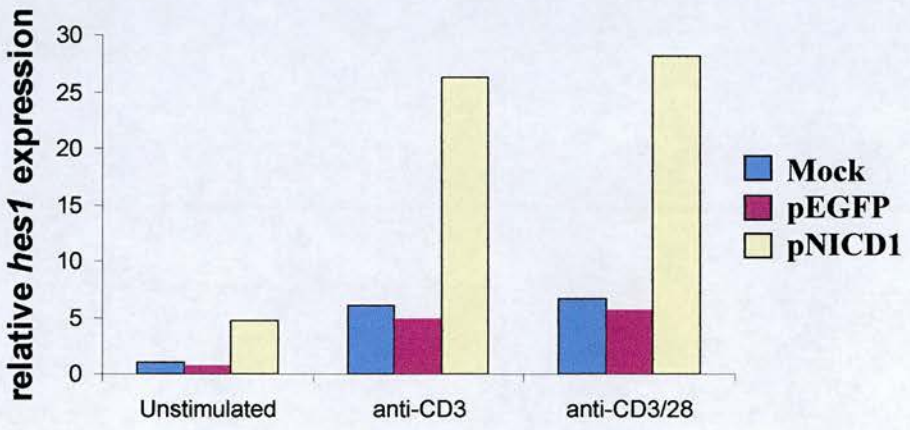


Figure 6.10: Relative *hes1* expression after pNICD1 transfection. $CD4^+$ T cells were isolated by MACs negative selection from human PBMCs. Cells were then transfected using the Amaxa system, either as a mock transfer or in the presence of pEGFP or pNICD1. RNA was collected after 48 hours of culture as unstimulated, anti-CD3- or anti-CD3/28-Ab treated cells. Real-time RT-PCR was used to assess expression of *hes1* relative to unstimulated mock transfected cells. A single experiment is shown.

Unfortunately, time restraints have only allowed this one initial experiment to be carried-out. The results are however, intriguing, particularly given that pNICD1 enhances cytokine secretion while repressing proliferation. Having only carried out this experiment once has meant that no suitable protein expression data has been generated. From this single experiment, some immunohistochemistry was prepared, using anti-myc antibodies. However, few cells were available for optimisation of the protocol and much background staining was evident, but some promising positive staining was evident.

6.5 Discussion

Although no direct conclusions can be drawn from the experiments detailed in this chapter, some interesting preliminary results were generated. Much of the adenoviral work was carried out at the beginning of this PhD, but was fraught with difficulty due to lack of effective reagents, rendering much of the data inconclusive. As such it must be stressed that the following discussion provides only speculative points based on the data and how interpretations may be made given current literature.

Notch signalling has been shown to affect $CD4^+$ T cell proliferation and cytokine secretion (97, 96). Much of this work has been established by prevention of Notch receptor cleavage (97, 96) or induction of Notch signalling using retrovirally transduced $CD4^+$ T cells (97). This section of work aimed to compliment previous chapters by stimulation of Notch receptor signalling, both ligand induced and by transfection of $CD4^+$ T cells with a constitutively active Notch receptor.

A DC enriched population over-expressing the Notch ligand Delta1 was used to activate $CD4^+$ T cells in an antigen specific fashion, utilising the DO11.10 TCR Tg T cells. The population of APCs generated was found to be rich in DC like cells (positive for CD11c, MHC class II and CD80), and could be infected with recombinant adenoviral vectors. A transfection efficiency of 80% was readily achievable using the protocol established. Attempts to demonstrate Delta1 transgene expression were of limited success, but were due to technical difficulties rather than being specific to a failure in transgene expression. To date, only low affinity,

polyclonal anti-Delta1 antibodies are available. Numerous attempts to achieve successful staining were made and in addition to using these antibodies, a recently developed Notch1-Fc fusion protein was used to try and demonstrate transgene expression, again unsuccessful. If a functional protein was expressed by the Ad-delta1 construct, minimal biological effect was mediated on $CD4^+$ T cells. Ad-delta1 DCs did enhance antigen non-specific $CD4^+$ T cell proliferation, but no significant difference was observed in antigen driven proliferation. Enhanced IFNγ and TNFα secretion reflected trends for increased [^{3}H] incorporation induced by all adenoviral treated groups. $CD4^+$ T cell IL-10 secretion was elevated when activation was by Ad-delta1 DCs, however this effect was not observed when LPS matured Ad-delta1 DCs were used.

Examination of the maturation state of dl70-3 and Ad-delta1 DCs revealed significantly enhanced expression of CD80/86 and MHC class II compared to non-virus treated. This would likely explain the trend for increased proliferation and cytokine levels observed when adenovirus treated DCs were used to activate $CD4^+$ T cells. Although this was not an objective of the project, it was reassuring that the DC population used did respond to viral infection, exhibiting that "danger signals" actually do functionally mature APCs.

Of note was the pronounced maturation of the Ad-delta1 DC population, even compared to the dl70-3 DCs. It has been reported that Notch signalling induced using Jagged1 can mature human DCs, in terms of surface marker expression and ability to enhance alloresponses (199). Greater maturation of the Ad-delta1 DCs discussed in

this chapter may indicate that the transgene is expressed and as a consequence the DC population is functionally matured by induction of Notch signalling.

Expression of high levels of co-stimulatory molecules may conceal minimal effects mediated by the transgene. Effects of Notch signalling on T cells have been shown to be sensitive to TCR signalling strength (97). Anti-CD3-Ab stimulated $CD4^+$ T cells have reduced cytokine secretion and diminished proliferative responses in the absence of Notch (97, 97). Addition of anti-CD28- as well as anti-CD3-Ab restores cytokine secretion (Chapter 4, page 104) and proliferation (97), indicating that Notch mediated effects may also be compensated for by co-stimulation. Adenoviral infection of DCs likely enhances TCR signalling and co-stimulation received by the T cell, masking any effect mediated by Delta1 induced Notch signalling. Recent work combining a technique developed for adenoviral infection of macrophages (personal communication, P Enrickson, Gene Transfer Group, Centre for Inflammation Research, University of Edinburgh) with the protocol established in this thesis for infection of dendritic cells may circumvent the problem of cell maturation upon encountering viral vectors. Transfection efficiency can be maintained using lower MOIs when virus particles are mixed with lipofectamine reagent (personal communication, A Roghanian, Gene Transfer Group, Centre for Inflammation Research, University of Edinburgh). Repeating the Ad-delta1 experiments using less mature DCs may generate clearer results.

IL-10 secretion by $CD4^+$ T cells induced by Ad-delta1 DCs was found to be higher than controls. This may be due to induced Notch signalling, particularly when

considered in the context of previous γ-secretase inhibitor experiments that highlighted the requirement of Notch signalling for production of this cytokine (Chapter 4.6, page 119). However, the maturation state of the Ad-delta1 DCs was also found to be significantly different from uninfected and control virus infected DCs. It is therefore equally feasible that the enhanced IL-10 secretion is due to differential co-stimulatory signalling.

Notch ligands have been over-expressed on APCs and used to activate T cells in two other systems (1, 105). The first used splenic DCs retrovirally transfected with human Serrate1 (1). Mice immunised with antigen pulsed Serrate DCs were rendered tolerant, which could be transferred to naïve mice by Serrate DC primed $CD4^+$ T cells (1). The activation state of the APC in this system was not addressed, nor was enhanced Notch signalling in the T cells demonstrated. This is not however, to detract from the fact that this Notch ligand was linked to generation of a regulatory population of T cells. It would be interesting to ascertain the mechanism of tolerance in this system, particularly if there was a role for IL-10.

A regulatory population of $CD4^+$ T cells was also generated in response to EBV-lymphoblastoid B cells over-expressing Jagged1 (105). These cells exhibited a Th3 phenotype, likely mediating suppression of cytotoxic activity via TGFβ (105). IL-10 secretion in this system was unaffected, as was the activation state of the transformed B cells (105). However, these systems used Jagged family ligands. The effect mediated by Delta induced signalling may be qualitatively different.

Use of Delta1-Fc fusion proteins has yielded conflicting data. Activation of $CD4^+$ T cells in the context of Delta1 induced signalling is reported to induce IL-10 secretion while inhibiting pro-inflammatory cytokine secretion (107). However, Delta1 signalling through Notch3 has been shown to induce Th1 polarisation (101), with high doses of Delta1-Fc reducing activation induced proliferation. In either case, a pronounced effect on $CD4^+$ T cells was observed, unlike those seen using Ad-delta1 DCs. A Delta1-Fc fusion protein has now been made commercially and if given the opportunity to revisit ligand induced Notch signalling, use of the fusion protein would be preferable to using Ad-delta1.

Nucleofection of primary human $CD4^+$ T cells with constitutively active Notch1 yielded some exciting preliminary results, particularly in the inhibition of proliferative responses. Cells transfected with pNICD1 did express higher levels of *hes1*, indicative of Notch signalling. These cells did not proliferate to the same extent as control groups, and this was not due to lack of IL-2 production. Transfection of a Jurkat cell line with NICD1 revealed inhibition of TCR induced NFAT/AP-1 reporter activity (99). Although NFAT itself may not be affected by Notch signalling as γ-secretase inhibition does not affect activity of this transcription factor (96, Chapter 4.7, page 123), repression of the aforementioned NFAT/AP-1 reporter is likely linked to prevention of AP-1 transcriptional activity. The intracellular domain of Notch1 is capable of suppressing AP-1 transcriptional activity (136). It might be speculated that TCR stimulation in the presence of Notch signalling sustains NFAT activity in the absence of AP-1, activating transcription of anergy associated genes (6), resulting in decreased proliferation. It is unlikely that the reduced proliferation

seen upon NICD1 transfection of human $CD4^+$ T cells is classical anergy since cells were still capable of secreting IL-2. It may simply indicate a switch to enhanced effector function at the expense of clonal expansion. In contrast, retroviral transduction of murine $CD4^+$ T cells with NICD1 was shown to enhance proliferation by up-regulating CD25 expression (97). Activation of T cell proliferation by Notch is also associated with lymphoproliferative disorders, where translocation events result in constitutively active receptors (78, 89). How this activation of proliferation relates to the data presented in this chapter is unclear, but may be a reflection of differences in transfection protocols or as with some Notch induced lymphomas, sustained expression of proliferation inducing receptors such as pre-TCR (89).

As with the retroviral transfer of NICD1 (97), induced Notch signalling in human $CD4^+$ T cells enhanced secretion of IL-2, even by unstimulated cells. Anti-CD3- and anti-CD3/28-Ab induced TNFα, IFNγ, IL-10, IL-4 and IL-5 secretion was also enhanced by NICD1 expression. Although Alder *et al* do not discuss the effect of NICD1 expression on cytokine secretion other than IL-2 (97), a similar system has reported that NICD1 does not affect IFNγ or IL-4 secretion (101). This effect was attributed to activity of NICD3, again utilising retroviral gene transfer. Enhanced cytokine secretion is likely to result from Notch enhanced NFκB activity (88, 96), being reduced by γ-secretase inhibition and constitutively active in NICD3 transgenic T cells. While IFNγ, IL-4 and IL-2 are known to have functional NFκB binding sites (154), IL-10 production may be refractory to NFκB activity (162). The fact that IL-10 secretion is also enhanced by pNICD1 suggests that an additional mechanism is

being employed. Regulation of IL-10 production by Notch was discussed in more detail previously (Chapter 4.8, page 126), but in summary, Notch signalling may regulate IL-10 gene transcription via HES1.

Continuation of the NICD1 work should prove fruitful, particularly if activity of NFκB, NFAT and Akt signalling pathways can be assessed in the context of Notch activation. However lack of detection of Delta1 protein would mean a more reliable system than using Ad-delta1, perhaps using the now commercially available Delta1-Fc protein, would prove more successful for *in vitro* studies. Had the Ad-delta1 experiments been more productive, the adenovirus would have allowed for adoptive transfer of Ad-delta1 DCs and assessment of Notch signalling and $CD4^+$ T cells activation *in vivo*.

7 Discussion

The hypothesis behind this thesis was that Notch signalling has a role in peripheral $CD4^+$ T cell differentiation. To address this, $CD4^+$ T cells were examined for the expression of Notch pathway components. The ability of stimulated $CD4^+$ T cells to proliferate and secrete cytokines (important in mediating effector function) was also assessed in the absence of Notch signalling. To further establish a role for Notch signalling in $CD4^+$ T cells, signalling was induced using both DCs over-expressing Delta-like1 and direct transfection of $CD4^+$ T cells with a constitutively active Notch1 construct.

Transcripts of both Notch receptors and ligands were detected in unstimulated and activated $CD4^+$ T cells, expression being differentially regulated following priming (Chapter 3.3, page 89). It was speculated that altered expression may reflect the requirement for particular Notch signals following TCR stimulation and pertained to development of effector function. Indeed the stimulation of a T cell by an APC bearing Notch ligand (184) might be likened to lateral induction observed in *Drosophila*, where Notch signalling occurs between two non-equivalent cell types (57), establishing the cell fate of the signal receiving cell. Stimulation of purified naïve $CD4^+$ T cells is associated with an increase in expression of the Notch target gene *hes1* (Chapter 3.3, page 89). This finding demonstrates that $CD4^+$ T cells are both receptive to Notch signals and must also be capable of delivering Notch signals.

It has been suggested that Notch mediates its effects on T cells by interaction with the TCR signalling pathway. Many surface receptors that have been shown to augment or antagonise TCR signalling are believed to be recruited to the immunological synapse where they are brought into close proximity to the TCR signalling molecules (2). The finding that Notch1 co-localises with CD4 on activated T cells (Chapter 3.4, page 113) strongly suggests that Notch receptors may physically associate with the TCR related signalling apparatus. An intriguing finding was recently reported documenting activation of Akt/PKB by Notch1 in a p56lck dependent manner, with Notch1 and p56lck co-immunoprecipitating (200). This adds credence to the idea that CD4 and Notch1 might co-localise, given the association of CD4 with p56lck (201). It is of interest to note that the fine line between T cell activation and anergy may relate to activity of lck relative to fyn, where higher fyn activity results in anergy (3). Additionally, Notch activation of Akt/PKB, a major target of CD28 signal transduction (202), would also support a role in augmenting T cell activation. Identification of a possible crossover point between Notch and CD28 signalling is of particular relevance in relation to the presented data pertaining to Notch and cytokine secretion.

Notch signalling was required for secretion of TNFα, IFNγ, IL-4 and IL-5 by anti-CD3-Ab stimulated $CD4^+$ T cells (Chapter 4.5, page 116). However, Notch signalling was not necessary for cytokine secretion when cells were stimulated with anti-CD28- in addition to anti-CD3-Ab. The ability of CD28 to substitute for Notch signalling in this context likely relates to the aforementioned activation of Akt by

these pathways. This supports the idea that Notch functions as a costimulator of T cells.

A key player in mediating Notch activity in a costimulatory capacity is likely to be NFκB. NFκB family members are known to direct transcription of inflammatory cytokines in T cells (203). Direct effects of Notch signalling on NFκB activity have been demonstrated in Notch3 transgenic mice and γ-secretase inhibitor experiments, demonstrating enhanced and impaired NFκB activity respectively (88, 96). The direct protein interactions linking Notch and NFκB have yet to be elucidated, but it seems highly plausible that Notch stimulation of Akt via lck would lead to activation of NFκB as it does in CD28 mediated costimulation. Based on this idea, it would be of value to examine Akt activity in anti-CD3-Ab stimulated $CD4^+$ T cells in the context of inhibited or induced Notch signalling (Figure 7.1).

Notch signalling may influence cytokine secretion more directly. The IL-6 promoter has been shown to contain a functional CBF-1 binding site (204). In this context, over-expression of CBF-1 inhibited secretion of IL-6. However, this paper did not address the ability of Notch signalling to convert CBF-1 from a transcriptional repressor to an activator. If this was found to be the case then perhaps the cytokines looked at in this study contain CBF-1 binding sites. It would be of interest to assess IL-6 secretion by $CD4^+$ T cells in the presence of the γ-secretase inhibitor to ascertain if this avenue would be worth pursuing.

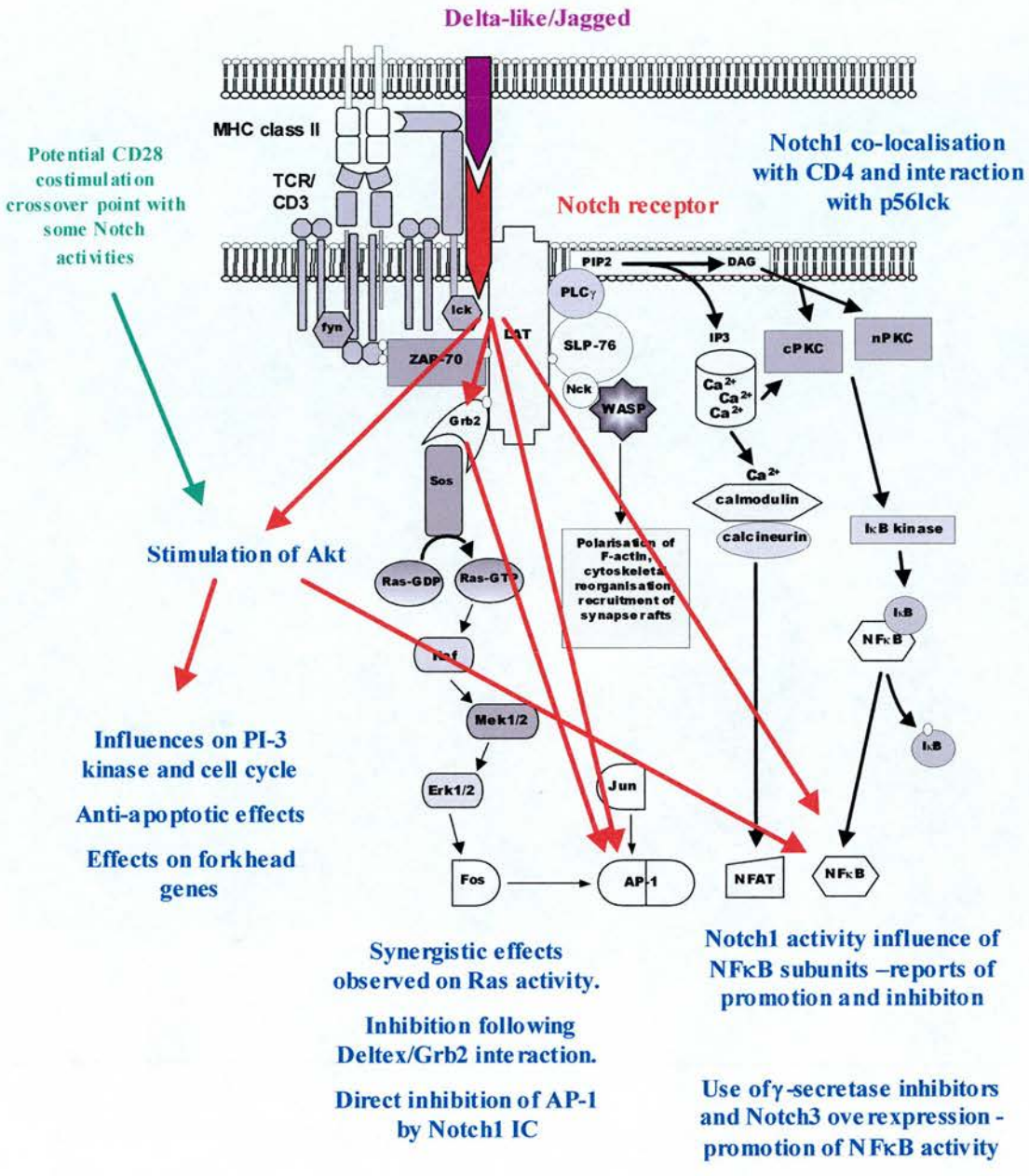


Figure 7.1: Potential interactions between Notch and TCR signalling. Red arrows show where Notch mediated signals interact with components of the TCR signalling apparatus, namely Akt, AP-1 and NFκB. The possible role of Notch as a costimulator of T cells is highlighted in green indicating where CD28 signalling overlaps with possible Notch targets.

A key finding that has shaped much of this thesis is the link between Notch signalling and IL-10 production (Chapter 4.6, page 119). Inhibition of Notch signalling was associated with a dramatic reduction in IL-10 secretion by stimulated $CD4^+$ T cells (even in the presence of anti-CD28-Ab costimulation). This was found to be an inhibition at a transcriptional level. The inability of CD28 signalling to compensate for the lack of Notch and restore IL-10 production likely reflects the profound differences between the IL-10 promoter and those of inflammatory cytokines (lack of functional NFκB and AP-1 binding sites) (162).

It was initially speculated that the lack of IL-10 mRNA may be due to a direct effect on transcription by Notch signalling. If this were the case, it might be expected that the IL-10 promoter would contain a CBF-1 binding site. Analysis of gene sequence showed that this was unlikely as no CBF-1 consensus site was identified, but a possible HES1 binding site was found (Chapter 4.6, page 119). Time constraints and a lack of technical skill meant that initial gel shift assays were unsuccessful in testing this possibility. However, this aspect of the work would be worth pursuing if a concrete connection between Notch signalling and IL-10 production is to be made.

The strong possibility that Notch and IL-10 are linked is evident in the data derived from experiments using T regulatory cells (Chapter 5, page 134). $CD25^+CD4^+$ T cells do secrete IL-10 in the system presented in this thesis. Where active regulation by $CD25^+CD4^+$ T cells was occurring, very large increases in *hes1* transcription were observed. Additionally, these cultures exhibited the largest amount of IL-10

production in relation to their degree of proliferation. These cells had already been shown to express high levels of *delta-like1*.

This begged the question as whether regulation was occurring via Delta-like1 induced Notch signalling in the target cell, as a direct effect of Notch signalling or due to induction of IL-10 secretion. Addition of high levels of γ-secretase inhibitor to $CD25^{+}CD4^{+}$ regulation cultures did not affect proliferation, but did result in a reduced ability to secrete IL-10 (Chapter 5.4, page 147).

Interestingly, *in vitro* generated Tr1 cells were also found to be high expressers of *delta-like1* (Chapter 5.6, page 158). This could perhaps relate to the ability of these cells to secrete high levels of IL-10. Indeed, unstimulated Tr1 cells expressed higher levels of *hes1* than non-regulatory counterparts, perhaps indicating that they have been subject to Notch signalling. It could be speculated that Delta-like1 expressed by Tr1 cells helps maintain the population's ability to secrete IL-10, but it may also provide a mechanism by which these cells could be generated (Figure 7.2). It has been suggested that naturally occurring $CD25^{+}CD4^{+}$ T regs may promote the generation of peripherally generated T regs (Tr1, Th3) (24).

Given the strong circumstantial evidence that Delta-like1/Notch interactions could be mediating the cell-cell contact dependent inhibition by $CD25^{+}CD4^{+}$ T regs, it was disappointing to discover that γ-secretase inhibitor attenuation of Notch signalling in $CD25^{+}CD4^{+}$ co-cultures did not prevent active regulation. Further investigation of Delta-like1/Notch interactions in this context would help to confirm this in the

author's own mind. Published literature detailing the use of a Delta-like1-Fc fusion protein demonstrated inhibition of T cell proliferation at the higher doses used in the study (101). Additionally, the presented data on transfection of human $CD4^+$ T cells with constitutively active Notch1 resulted in reduced proliferation of these cells upon polyclonal stimulation. This lends credence to the idea that Notch signalling may be a cell-cell contact mechanism that could be utilised in active regulation by $CD25^+CD4^+$ T regs. Pre-treatment of $CD4^+$ T cells with γ-secretase inhibitor before use in $CD25^+CD4^+$ T reg co-cultures could rule out the possibility that suppression is due to very early rather than prolonged Notch signalling. Use of RNAi technology to prevent expression of Delta-like1 by T cells may prove useful in determining if Notch signalling induced by this ligand mediates suppression of $CD4^+$ T cell proliferation. In addition, an in-house inducible *delta-like1* transgenic mouse has been developed. The *in vitro* responses of $CD4^+$ T cells derived from this mouse are currently being investigated, but it would be interesting to compare these cells with $CD25^+CD4^+$ T regs or Tr1 cells.

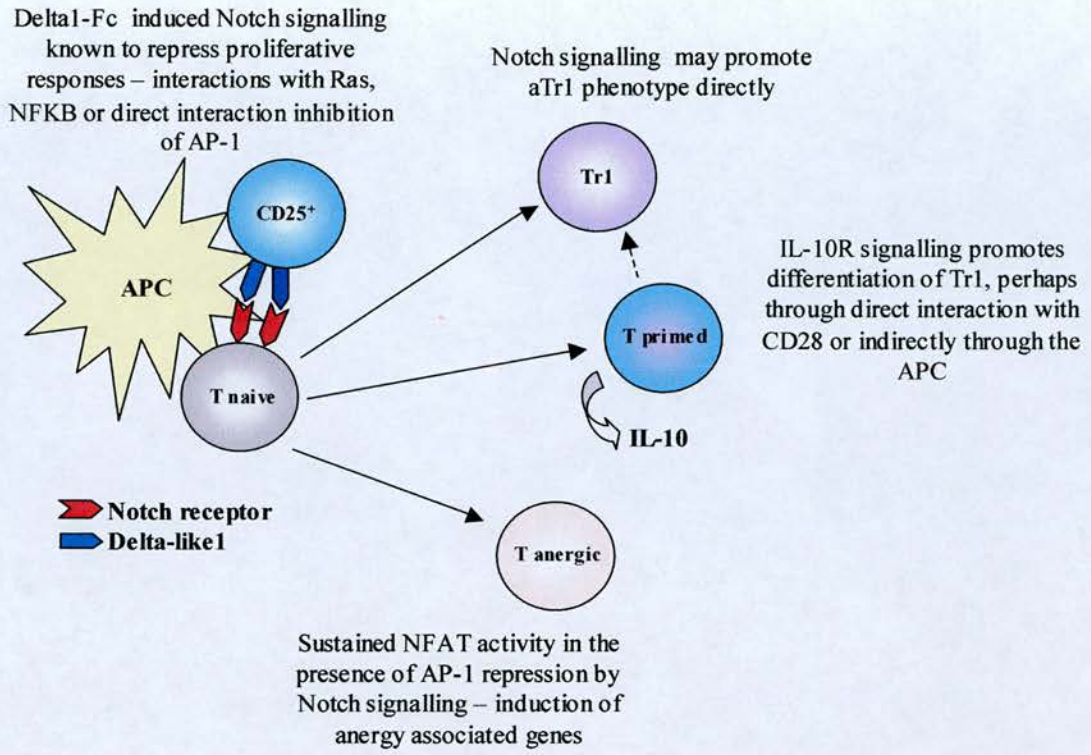


Figure 7.2: Notch in active regulation by $CD25^+CD4^+$ T regs. $CD25^+CD4^+$ T regs induce a Notch signal in target naïve $CD4^+$ T cell undergoing priming. High levels of transduced Notch signals may result in anergy or allow generation of Tr1 cells directly or by inducing IL-10 production by an intermediate T primed cell, then when antigenic stimulus persists, IL-10 induces Tr1 differentiation.

Reconciliation of the idea of Notch as a costimulator of $CD4^+$ T cells with the idea that it may also act to suppress their function is difficult. However, in an attempt to do so, it might be proposed that Notch signalling in $CD4^+$ T cells would initiate receptiveness to polarisation stimuli (Figure 7.3). As such a biphasic system is suggested where initial low level Notch activity facilitates activation of the T cell. Once a certain threshold of Notch activity is reached, signalling promotes a switch in cell activity from clonal expansion to differentiation of effector function. Regulatory populations of T cells may preferentially utilise the Notch pathway as a way of shaping responses of target cells, perhaps by increasing their receptiveness to yet unidentified signals from T regs. This may be reflected in the generation of Tr1 like cells by $CD25^+CD4^+$ T cells.

Interaction with an APC bearing Notch ligands would recruit Notch receptors to the immunological synapse. Here Notch may initially function to augment TCR and CD28 signalling, based on lck dependent Akt activation and data pertaining to enhanced NFκB activity (200, 88, 96). Inhibition of Nur77 would also support survival of $CD4^+$ T cells, preventing TCR induced apoptosis (102), possibly pertaining to later differentiation into memory cells. Induction of IL-2 and CD25 would also support clonal expansion of the Th0 population (97).

The function of Notch signalling is closely related to cell cycle. G2 cell cycle arrest is required for Drosophila neural cell fate determination (205). The effect of Notch signalling following cessation of cell division may be different based on differences in degradation resulting in disruption of signalling mediator equilibriums. Slowing of clonal expansion due to limiting IL-2 levels may precede development of effector function. Fully differentiated Th1 and Th2 cells are poorer sources of IL-2 than Th0 cells. Indeed, after 48 hours of in vitro culture, it is often difficult to detect IL-2 in supernatants. Perhaps during the intermediate stage between being a Th0 and terminally differentiated Th1/Th2 cell, slowing of proliferation switches the role of Notch signalling in the cell. In this context Notch signalling may facilitate receptiveness to particular polarisation stimuli, or might reinforce differentiation through chromatin remodelling. The interplay between Notch signalling and histone acetyltransferases and deacetylases is well documented and may link into epigenetic views on helper cell polarisation (206).

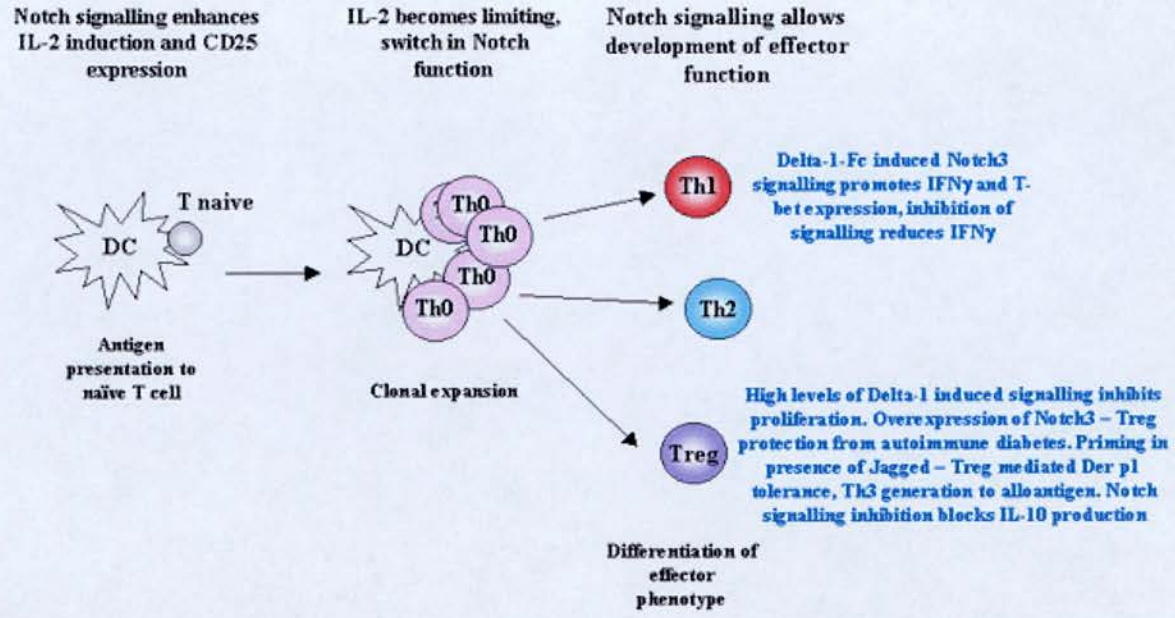


Figure 7.3: A biphasic role for Notch in CD4$^+$ T cell function: Priming of naïve CD4$^+$ T cells in the context of Notch signalling would promote clonal expansion through augmentation of IL-2 secretion when costimulation is limiting, and stimulating expression of CD25. Population proliferation begins to slow as Th0 cells utilise the available IL-2. Alterations in cell cycling enhances stability of Notch signalling components allowing the primed T cell to become more receptive to polarisation stimuli such as cytokines, signalling from Th1/Th2/T reg signalosomes or costimulation. Notch signalling may then function to fortify helper/regulatory cell differentiation.

Studies into the subversion of immune responses by pathogens have advanced the understanding how the immune system functions. Using this other perspective to study Notch will likely be effective in understanding the role of this developmental pathway in normal operation of the immune system. The EBV protein EBNA2 shares much homology with the intracellular domain of Notch. Many of the mechanisms by which Notch signalling directs its influence have been shown to integrate with EBNA2 signalling, including binding of CBF-1 (207), inhibition of Nur77 induced apoptosis (208) and acetylation of histones (209). Of additional interest, particularly in light of the IL-10 data presented in this thesis, is the identification of an EBNA2 binding consensus in the human IL-10 promoter (210). Although this study found

that EBNA2 was not required for LPS induced IL-10 secretion in a monocyte cell line, it does raise the possibility that EBNA2/NICD may regulate lymphocyte secretion of IL-10 more directly rather than expected.

It had been said that a PhD generates more questions than it answers, and this is certainly true of Notch and $CD4^+$ T cell function. Deciphering the current data pertaining to this aspect of T cell biology and that still to come will likely highlight the complexity of the system. Having numerous receptor and ligand homologues providing qualitative and quantitative signals is complicated, but identification of numerous molecules that can regulate these signals will no doubt add numerous new levels of control to the system. Examination of these positive and negative regulators in the context of mature T cell function will likely prove fruitful as has been the case in thymocyte studies. What is clear though is that much work still remains to be done!

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